1-1-2003

Interactions of rationally designed multiple-aptamer constructs

Becky Ann Stodola
Iowa State University

Interactions of rationally designed multiple-aptamer constructs

by

Becky Ann Stodola

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biochemistry

Program of Study Committee:
Marit Nilsen-Hamilton (Major Professor)
Gloria Culver
Victor Lin

Iowa State University
Ames, Iowa
2003

Graduate College
Iowa State University

This is to certify that the master's thesis of

Becky Ann Stodola

has met the requirements of Iowa State University

Signatures have been redacted for privacy

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

DNA and RNA aptamers are becoming increasingly popular as biotechnological tools and possible therapeutic molecules, as they can often match or surpass the binding affinity and specificity of antibodies for their target in a much smaller molecule that is often better suited to experimental conditions. Aminoglycoside antibiotics were among the first targets for aptamers due to their high natural affinity for RNA, binding to many different sequence and structural motifs, resulting in a "selective" affinity for RNA which is less than specific, yet not quite broad enough to qualify as nonspecific binding. This selective binding allows aminoglycosides to bind to aptamers selected against other ligands, potentially altering the behavior of aptamer constructs containing aptamers not specifically targeted towards aminoglycosides. A selective aptamer binding enhancement by neomycin and a smaller increase by tobramycin is reported here when two aptamers are joined together into Cis-Linked Aptamers for Microanalytical Procedures (CLAMPs), even when the aptamers were not designed to bind aminoglycosides. This results in allosteric binding behavior in these CLAMPs, where selective binding by aminoglycosides increases the binding ability of one of the aptamers. This allostery may be the result of these aminoglycosides binding to a portion of the aptamer constructs that is distinct from the regulated aptamer, stabilizing the structure of that portion of the construct so it does not interfere with the folding and binding of the regulated aptamer. This restores higher binding in an aptamer construct in which additional sequence adds to folding options and interferes with aptamer binding. In this way; aminoglycosides may act as generic allosteric activators for many different aptamer

constructs.

CLAMPs can also be used to link different species of fluorescent mesoporous silica nanospheres. In this way the fluorescent nanospheres can form optical logic gates, where two species of nanosphere are bound close enough by the CLAMP to produce fluorescence resonance energy transfer (FRET), an unique output from the input of the two nanospheres.

CHAPTER 1: GENERAL INTRODUCTION

Introduction

RNA and DNA have long been known to specifically bind proteins and small molecules during cellular processes such as replication, transcription, and translation. Due to this highly interactive behavior of either form of oligonucleotide, it should be no surprise that nucleic acid sequences can be found and isolated that bind to a wide range of molecules and can be utilized in a variety of biotechnological applications. A specific sequence of RNA or DNA that binds a certain molecule with high specificity and high affinity is called an aptamer and frequently consists of between 30 and 50 nucleotides. Aptamers can be selected by an *in vitro* procedure in which a random sequence library of oligonucleotides is selected for binding to the desired target molecule through multiple rounds of binding to an affinity column, amplifying the binding sequences, and finally cloning and sequencing the resulting set of aptamer sequences (*1, 2*). This selection method is also called SELEX, or Systematic Evolution of Ligands by EXponential enrichment (*2*), and can be used with a variety of DNA, RNA, or modified oligonucleotide sequence libraries and experimental conditions to facilitate discovery of aptamers suited for use at different temperatures, buffers, or cellular conditions.

Development of aptamer applications has progressed remarkably, with over 100 published DNA, RNA, and modified RNA aptamer sequences to different small molecules and proteins in the past 10 years. Aptamers are currently being tested for therapeutic applications such as inhibiting NS3 protease of the hepatitis C virus (*3*) or inhibiting the human transcription factor NF-κB for control of inflammation (*4, 5*). An aptamer inhibitor of Platelet

Derived Growth Factor is currently being tested as a cancer treatment (*6, 7*). Promising new aptamer-based alternatives for inhibiting thrombin for anticoagulation and vascular disease therapy are also being tested (*8-12*).

While aptamers directed at proteins are increasing in popularity as potential new therapeutic tools, aptamers specific for small molecules are better suited to detailed examinations of the behavior of aptamers. Small molecules are stable in a wider variety of conditions and do not require proper folding like proteins. Small molecule ligands are also often completely enclosed in the aptamer binding pocket (*13-17*), reducing possible interferences with other portions of a complex aptamer construct containing additional sequence elements. The exact structure of an aptamer sequence is difficult to predict until an X-ray crystal structure or NMR solution structure has been determined, especially with RNA, which has many possibilities for folding and tertiary interactions. Even after a crystal or solution structure has been determined, aptamers likely have other folded states in solution that remain unobserved, balanced in a dynamic folding equilibrium (*18, 19*). The existence of this folding equilibrium suggests that more valuable aptamer constructs could be designed if their binding and interactions were better understood.

Aptamers are even more valuable as biotechnological tools when their binding ability is combined with additional functionalities. Two aptamer sequences can be combined into dual aptamer constructs such that the binding of one ligand may either increase or decrease binding of the second ligand. The *in vitro* selection method can be modified to directly select for aptamer constructs with the desired behavior, sometimes called an allosteric selection. An aptamer inhibitor of the DNA repair enzyme formamidopyrimidine glycosylase was selected in

this manner such that its inhibitory effects are reduced by neomycin binding (*20*). Work done with ribozymes, RNA molecules with a catalytic ability, demonstrate several regulation schemes that could be applied to aptamers. Allosteric selection successfully identified a ribozyme dependent upon a small molecule ligand for catalytic ability (*21*). Even without employing allosteric selection, regulated ribozymes can be created by rational modular design, where separate catalytic or ligand-binding sequences are joined by a linking sequence to form a ribozyme with a more complex behavior. In this manner, ribozymes have been constructed that are stimulated (*22*) or inhibited (*23*) by an effector molecule, or even dependent upon binding of two effector molecules for maximum catalysis (*24*). While this shows incredible potential for rationally designing regulated ribozymes and aptamers, not all designs are this successful. Often multiple ribozyme constructs are made, and only a few are regulated by their effector molecule (*22, 25, 26*). The linking region between catalytic or ligand binding domains appears to be essential to create successful regulation. To aid in modular rational design, an allosteric selection step can be performed after initial design to select for a linker that creates an effective, regulated ribozyme (*26-28*).

While the only published allosteric aptamer was created using allosteric selection (*20*), we have begun to utilize a rational design strategy for engineering allosteric aptamers. This approach has shown some measure of success in designing allosteric ribozymes, so rational design may also be useful for creating regulated aptamers.

Thesis Organization

Chapter 2 contains the journal article manuscript pertaining to the selective aptamer binding enhancement (generic allosteric activation) caused by aminoglycoside antibiotics. This paper is in preparation for submission to Biochemistry for publication. I will be the first author on this paper, and Marit Nilsen-Hamilton will be the second and only other author, being the principal investigator on this project. Chapter 3 details the efforts to date to utilize small molecule dual aptamer constructs for linking fluorescent silica nanospheres for use as a potential optical logic gate and data storage biotechnology. This project is a collaboration with Victor Lin's group in the Chemistry department of ISU, who proposed the project. General conclusions follow these two separate projects in Chapter 4.

CHAPTER 2: SELECTIVE APTAMER BINDING ENHANCEMENT BY AMINOGLYCOSIDES

a paper to be submitted to Biochemistry

Becky A. Stodola and Marit Nilsen-Hamilton

Abstract

Many aptamers have been selected towards aminoglycoside molecules due to their natural affinity for nucleic acids, but this may cause later problems of specificity due to their ability to bind many different sequence and structure motifs. Aminoglycoside binding of nucleic acids may be better described as selective binding due to the wide range of sequences recognized. Here we combine two RNA aptamer sequences into a single molecule to form Cis-Linked Aptamers for Microanalytical Procedures (CLAMPs). Several aminoglycosides are able to bind selectively to RNA CLAMPs that contain non-aminoglycoside directed aptamers and that influence the binding ability of aptamers in these constructs. We propose that aminoglycosides have an allosteric effect upon binding of these dual aptamer constructs to their ligands, increasing the binding of one aptamer by selectively binding other parts of the construct and removing them from the folding options in the dynamic folding equilibrium of these complex RNA molecules.

Introduction

Aminoglycoside antibiotics are popular molecular targets for aptamers due to their high affinity for binding RNA and DNA and potential use as therapeutic treatments utilizing aptamer inhibitors. The high nucleic acid binding potential of aminoglycoside molecules is

apparent from the many different sequences and structures recognized by aminoglycosides such as neomycin, tobramycin, and kanamycin. Natural RNA sequences that aminoglycosides bind specifically include the A site of ribosomal 16S rRNA (*29-35*), HIV regions TAR (*36*) and RRE (*37*), the 5' untranslated region of thymidylate synthase mRNA (*38*), $tRNA^{Asp}$ (*19*), $tRNA^{Phe}$ (*39*), and a splicing regulatory element in exon 10 of the tau protein (*40*). Tobramycin has even been shown to bind to a polymeric designed oligonucleotide with a poly(rI)poly(rC) sequence (*41*). Although binding of aminoglycosides to a certain nucleic acid sequence is often reported as specific binding, the wide range of sequences recognized suggests that overall this would be better described as selective binding, where many different sequences form possible binding sites.

Although most studies have focused on the binding properties of specific RNA sequences for aminoglycosides, it appears that the RNA structure is more important than the nucleic acid sequence (*38*). Many of the RNA sequences that aminoglycosides bind involve distorted helical regions, including internal bulges (*35-38, 42*), mismatch nucleotide pairs (*37, 43, 44*), and short stem loop structures (*40, 42, 44, 45*). Many selected RNA aptamer sequences also form stem loop structures, including aminoglycoside aptamers (*35, 43, 45-47*). Some researchers even suggest that a non-A form RNA structure may be sufficient to bestow aminoglycoside binding (*42, 47*).

Aminoglycosides have been shown to stabilize triplex RNA and DNA (*48*) and enhance thermal stability of the A site of 16S rRNA (*34*). The inhibitory effects of aminoglycosides on functional RNA are often believed to be due to distortions caused by their binding (*30, 31, 36, 49*). Aminoglycosides are also known to displace metal ions upon

binding of several RNA sequences (*39, 50, 51*). Small RNA molecules such as aptamers are proposed to have multiple stable structures, which likely exist in a dynamic folding equilibrium, where any given RNA molecule will shift between these stable structures. If aminoglycosides bind and stabilize a minor structural form, they shift this dynamic folding equilibrium towards the stabilized structure (*18, 19*).

Aminoglycosides can affect the structure and function of RNA aptamers such that binding to the target molecule is reduced by an aminoglycoside such as neomycin (*20*). An allosteric selection procedure was used to select this regulated aptamer sequence, and kanamycin did not cause the same effect, so this appears to be a specific allosteric effect. However, when aminoglycosides are used the specificity of these effects may still be questionable due to their high natural affinity for nucleic acids. A higher concentration of an alternate aminoglycoside may cause the same effect as the specified ligand, so claims of specificity must be examined carefully.

Here it is reported that aminoglycosides can produce an allosteric binding effect in rationally designed Cis-Linked Aptamers for Microanalytical Procedures (CLAMPs) that contain two aptamer sequences in a single RNA molecule. In these CLAMPs, binding of an aminoglycoside increases the binding ability of one of the aptamers. This increase in aptamer binding was shown to occur with dual aptamer constructs that contained various aptamer pairs and linkers, even when there was no aminoglycoside aptamer present. The binding of a single aptamer to its target is not influenced in this manner, so we believe the aminoglycosides act upon the additional sequence not already involved in specific binding to its ligand. We propose that the aminoglycosides bind selectively to this extra RNA segment, stabilizing its

structure, and allowing it less freedom to interfere with folding of the binding aptamer, thus increasing the binding ability of the aptamer. The aminoglycosides neomycin and tobramycin were both shown to produce this effect although the neomycin effect was larger than for tobramycin. This may suggest the use of aminoglycoside antibiotics to aid in folding and ligand binding of larger aptamer constructs containing more than a single aptamer sequence. This does complicate working with aminoglycoside aptamers, since binding behaviors are likely to be a complex mixture of specific and selective binding effects.

Materials and Methods

Materials. Neomycin, tobramycin, adenosine-5'-triphosphate (ATP), and theophylline were purchased from Sigma. Tris base, sodium chloride, and magnesium chloride were purchased from Fisher Scientific.

RNA aptamer and CLAMP sample preparation. RNA secondary structures were predicted by the Mfold program (*52, 53*). Linear DNA templates and primer oligos were designed containing a T7 promoter before the aptamer or CLAMP sequence and were synthesized by the DNA Sequencing and Synthesis Facility of Iowa State University. DNA templates were PCR amplified on a PTC-150 Mini Thermal Cycler (MJ Research) using *Taq* DNA polymerase (Promega) and deoxynucleotide triphosphates (MBI Fermentas). The double stranded DNA templates for the RNA aptamers and constructs were transcribed using T7 RNA polymerase and ribonucleotides (Promega), RNaseOUT ribonucleotide inhibitor (Invitrogen), and [α^{32}P]rCTP (ICN). An AmpliScribe T7 Transcription Kit (Epicentre Technologies) was used according to manufacturer's instructions for high yield production of

RNA for equilibrium dialysis. Aptamers and CLAMPs were purified using BioSpin 30 Chromatography Columns (BioRad) to remove free NTPs.

Column affinity aptamer binding assay. ATP agarose with a C8 linkage (Sigma) was used to measure ATP binding. Cyanogen bromide activated sepharose (Pharmacia Biotech) was linked to the amino groups of neomycin to produce an affinity matrix to measure neomycin binding. Following manufacturer's instructions for preparation of the sepharose, 1g dry gel powder was swollen and coupled in a 1mM neomycin solution at pH 7.6 to obtain approximately 2 umole neomycin coupled per 1 mL of gel. Remaining active groups were then blocked by a secondary coupling with a 0.2M glycine solution.

Affinity column binding experiments were performed using empty micro biospin columns (BioRad) containing approximately 100ul of affinity matrix. The column was washed with 10 column volumes (1mL) of buffer A (50mM Tris, 250mM NaCl, 5mM $MgCl_2$, pH 7.6 at 23°C) before loading ^{32}P-labeled aptamer or CLAMP in buffer A. After the radiolabeled RNA sample was added to the column, the column was washed with 20 column volumes (2mL) of buffer A, then the bound aptamer was eluted with 10 column volumes (1mL) of buffer A containing 10mM of the ligand. Wash and elution fractions were measured by scintillation or Cerenkov spectroscopy. When Cerenkov spectroscopy was used, the affinity matrix was also counted to check for aptamer that remained bound to the column after the RNA was eluted with the ligand.

Gel mobility shift aptamer binding assay. ^{32}P-labeled CLAMPs were mixed with ATP agarose at 23°C then resolved by 12% native polyacrylamide gels in TBE buffer (89mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.5 at 23°C) to separate the bound from the unbound

CLAMPs. Gels were dried and the bands of unbound CLAMP were imaged and quantified by a Model 400A PhosphorImager (Molecular Dynamics).

Equilibrium dialysis assay. Equilibrium dialysis experiments utilized Equilibrium Dialyzers and 5kDa regenerated cellulose membranes (Nest Group). CLAMPs are between 65 and 83 nucleotides in length and should not be able to pass the membrane while the radiolabeled ATP can equilibrate across the membrane. One chamber of each dialyzer contained buffer A, CLAMP, and the variable ligand, and the other chamber contained buffer A, [γ^{32}P]ATP (ICN), and the variable ligand. The concentration of the ligand was varied in the different dialyzers to produce data for the effect of a gradient of ligand concentrations. The dialyzers were allowed to equilibrate overnight, and then samples were removed from each side of the membrane for ATP concentration measurement by scintillation spectroscopy. The percentage of ATP bound CLAMP was calculated from the ATP distribution across the chambers.

T1 cleavage protection assay. Samples of Theophylline-ATP CLAMP 613 were prepared with or without 1mM theophylline. From 0 to 0.05U/ul of ribonuclease-T_1 (Calbiochem) were added to the samples immediately before loading and running at 23°C on a native 20% polyacrylamide gel in TBE buffer (89mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.5). The CLAMP was exposed to the ribonuclease for 5 min before loading on the gel. Gels were dried and the bands of RNA fragments were then imaged and quantified by a Model 400A PhosphorImager (Molecular Dynamics). The cleavage patterns were compared to determine cleavage protection by theophylline aptamer binding.

Significance of all results were determined using a critical double tailed t test for all

values of p reported, calculated with a certainty of 95%.

Results and Discussion

RNA Neomycin/ATP dual aptamer CLAMPs were designed using a neomycin binding sequence (*44*) and an ATP binding sequence (*14, 54*) (Table 1). The two ligands have very different structures (Figure 1), so binding of the opposite ligand should be minimized. The most stable predicted secondary structure of each of these aptamers (Figure 2a & c) resembles the published tertiary structures. CLAMPs were made with the two aptamers in either order with two uracil nucleotides as a linker, and also neomycin 5' to ATP 3' with a ten uracil linker (Table 1). Both aptamers are predicted to fold independently as the separate aptamers (Figure 2) when separated by two uracil nucleotides in the CLAMPs (Figures 3a & 4a), where they could not in constructs without the linker. The CLAMP with the ten uracil linker was produced to test the effect of a larger separation between the aptamers on their binding behavior. For each of these CLAMPs, there are at least 15 possible stable secondary structures (Figures 3 & 4) predicted by Mfold (*52, 53*), so a population of RNA molecules are likely to form a variety of conformations at any given time, where any one molecule can switch between stable structures.

In the Neomycin/ATP CLAMPs, the presence of neomycin increased the binding ability of the ATP aptamer end. This was shown using column affinity experiments (Figure 5), where the overall activity of the RNA preparations varied, but the ATP binding always increased when 1mM neomycin was present. Gel binding assays (Figure 6) were also used to examine the effect of varied neomycin concentrations on ATP binding by the Neomycin-uu-

ATP CLAMP 609 and ATP-uu-Neomycin CLAMP 610. From these gel binding assays, the enhancement of ATP binding is seen to be concentration dependent, with some increase in ATP binding seen with 100uM or even 10uM neomycin. There was a logarithmic relationship between the neomycin concentration and the ATP binding. While this method is valuable for comparing many samples and conditions, measuring the unbound CLAMP in this way did not indicate how much of the CLAMP was bound to the ATP-agarose, which would be a more valuable measurement. There also appeared to be more error in gel binding data than when using column affinity. For example, in Figure 6a, the data points at a 1mM neomycin concentration appear to be erroneous for both bands of unbound CLAMP in the gel.

While the most commonly used assay for testing aptamer binding is affinity chromatography, and gel binding has also proven useful, both of these assays involve a solid phase of some kind to which the aptamer must bind or move through. Equilibrium dialysis was used to test the binding of the allosteric CLAMPs in solution (Figure 7). This method was surprisingly difficult to use for testing aptamer binding because it required large amounts of RNA and returned high errors. With some effort, however, data was obtained which supports the increase in ATP binding caused by the binding of neomycin (Figure 7). This data shows ATP binding to be about doubled with 120uM neomycin, which is about 10 times the RNA concentration, still a relatively low concentration to be producing this effect.

RNA theophylline/ATP CLAMPs were designed with a theophylline binding sequence (*16, 55*) 5' to an ATP binding sequence (*14, 54*), with (CLAMP 614) or without (CLAMP 613) 10 uracil nucleotides as a linker between the two aptamers (Table 1). The structures of theophylline and ATP (Figure 8) are different enough to reduce the possibility of binding the

opposite ligand. Mfold secondary structure predictions (*52, 53*) return over 20 possible conformations for each CLAMP, consistent with the expected conformational flexibility and dynamic folding properties of small RNA molecules. The most stable structure for each construct resembles the structures of the single aptamers joined together (Figure 9), much like the neomycin/ATP CLAMPs.

The addition of 1mM neomycin to the affinity column binding buffer increased the ATP binding of RNA theophylline/ATP CLAMPs 613 and 614 (Figures 10 & 11). The presence of tobramycin also had a small effect. The same concentrations of neomycin or tobramycin did not affect the binding of the RNA ATP aptamer sequence by itself (Figure 12). This suggests that these aminoglycosides may act only upon the sequence 5' to the ATP aptamer end. In the CLAMPs, the presence or absence of the 10 uracil linker does not appear to change this increase in ATP binding due to aminoglycosides. These results suggest that the site of aminoglycoside binding is either the theophylline aptamer or the extra sequence in the CLAMP beyond the ATP aptamer. The theophylline aptamer contains structural motifs known to have aminoglycoside binding properties, a stem loop (*35, 43, 45-47*) and an internal bulge (*35-38, 42*), so it is no surprise that neomycin (and perhaps tobramycin) can bind to this end of the CLAMP. It is interesting to note that aminoglycosides increased the percentage of binding CLAMP (Figures 10 & 11) to a level closer to the original binding of the RNA ATP aptamer (Figure 12). This argues that the aminoglycosides restore ATP binding ability that was lost by the ATP aptamer when it was incorporated into the CLAMP. The additional nucleic acid sequence in the CLAMP may interfere with the proper folding of the ATP aptamer.

Because aminoglycosides may increase ATP binding by stabilizing the structure of the second aptamer in the CLAMP, it may be expected that theophylline will also stabilize its aptamer, and thereby increase ATP binding. However, this does not appear to be the case. In column affinity experiments 1mM theophylline did not affect the ATP binding of the Theophylline-ATP CLAMP 613 (Figure 10) or the Theophylline-10u-ATP CLAMP 614 (Figure 11a). The Theophylline-10u-ATP CLAMP 614 affinity column binding data show a possible slight decrease in ATP binding upon 1mM theophylline addition (Figure 11a, double tailed t test, $p = 0.03$). However, in equilibrium dialysis experiments theophylline did not affect the ATP binding ability of either Theophylline-ATP CLAMP 613 or Theophylline-10u-ATP CLAMP 614 (Figure 13). Together, these results do not support the conclusion that theophylline alters ATP binding activity in the CLAMPs.

With no present access to a theophylline affinity matrix, determining that theophylline is actually binding to the Theophylline/ATP CLAMPs is difficult. The proper theophylline aptamer sequence (*16, 55*) is present in the sequence, and Mfold (*52, 53*) predicts that it should fold as required for binding its ligand, but still there may be some interference that prevents theophylline binding. To support our assumption of theophylline binding, ribonuclease-T_1 cleavage assays were performed to test for cleavage protection provided by theophylline binding. There are several changes in the band intensity patterns between the samples with or without theophylline (Figure 14). Quantification of the band intensity changes showed that the overall trend upon addition of theophylline was an increase in the more slowly migrating RNA fragments and a decrease in the more rapidly migrating RNA fragments (Table 2), indicating that theophylline bestowed partial protection from T_1 cleavage.

This supports our assumption of theophylline binding to the Theophylline-ATP CLAMP 613. Once a theophylline affinity matrix can be obtained, affinity column experiments will be performed to test for theophylline binding.

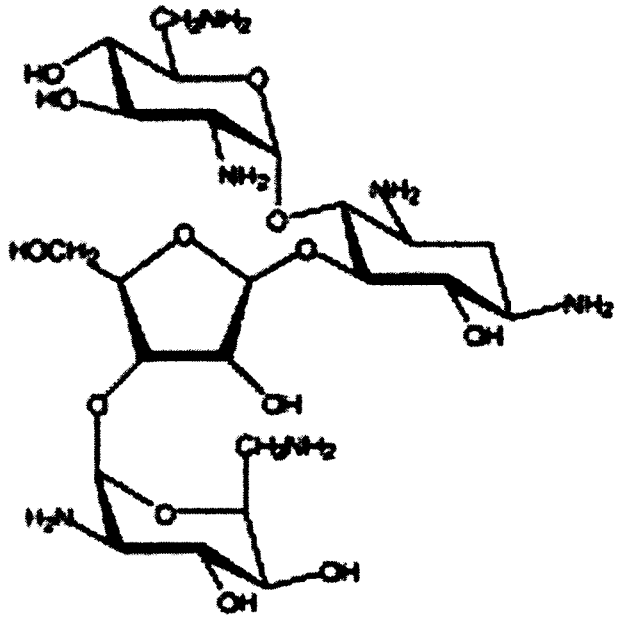
The effect of neomycin and tobramycin on the Neomycin/ATP and Theophylline/ATP CLAMPs appears to be an allosteric enhancement of ATP binding ability caused by the selective binding of aminoglycosides. We propose that when the aminoglycosides bind to the neomycin or theophylline aptamer, the aptamer structure is stabilized (Figure 1a & b), potentially reducing its interference with the ATP aptamer end of the CLAMP. This decreases the fraction of CLAMP in states with misfolding of the ATP aptamer, such as the structures shown in Figure 2d & e for Neomycin-uu-ATP CLAMP 609. Thus, aminoglycoside binding is proposed to shift the dynamic folding equilibrium towards conformational states where the ATP aptamer is properly folded for binding, such as the structure shown in Figure 2a for Neomycin-uu-ATP CLAMP 609. This shift in the folding equilibrium would explain the increase in the overall ATP binding of the CLAMP. In this way the aminoglycosides perform a chaperone role in aiding proper folding of the ATP aptamer within the CLAMPs.

Table 1: RNA Aptamers and CLAMP Design[a]

Aptamer / CLAMP Name	Number
Sequence	
Neomycin Aptamer (*44*)	601
ggccugggcgagaaguuuaggcc	
Theophylline Aptamer (*16, 55*)	603
ggcgauaccagccgaaaggcccuuggcagcguc	
ATP Aptamer (*14, 54*)	605
gggguugggaagaaacuguggcacuucggugccagcaaccc	
Neomycin-uu-ATP CLAMP	609
ggccugggcgagaaguuuaggcc uu gggguugggaagaaacuguggcacuucggugccagcaaccc	
ATP-uu-Neomycin CLAMP	610
gggguugggaagaaacuguggcacuucggugccagcaaccc uu ggccugggcgagaaguuuaggcc	
Neomycin-10u-ATP CLAMP	612
ggccugggcgagaaguuuaggcc uuuuuuuuuu gggguugggaagaaacuguggcacuucggugccagcaaccc	
Theophylline-ATP CLAMP	613
ggcgauaccagccgaaaggcccuuggcagcguc gggguugggaagaaacuguggcacuucggugccagcaaccc	
Theophylline-10u-ATP CLAMP	614
ggcgauaccagccgaaaggcccuuggcagcguc uuuuuuuuuu gggguugggaagaaacuguggcacuucggugccagcaaccc	

[a] Linkers are underlined

(a)



(b)

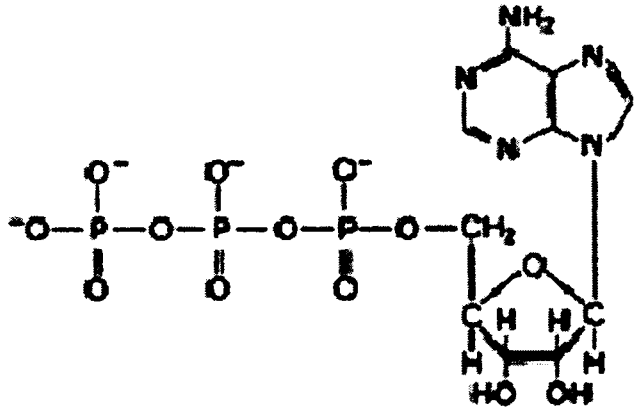


Figure 1: Neomycin and ATP structures. The structures of (a) neomycin and (b) adenosine 5'-triphosphate (ATP) are shown.

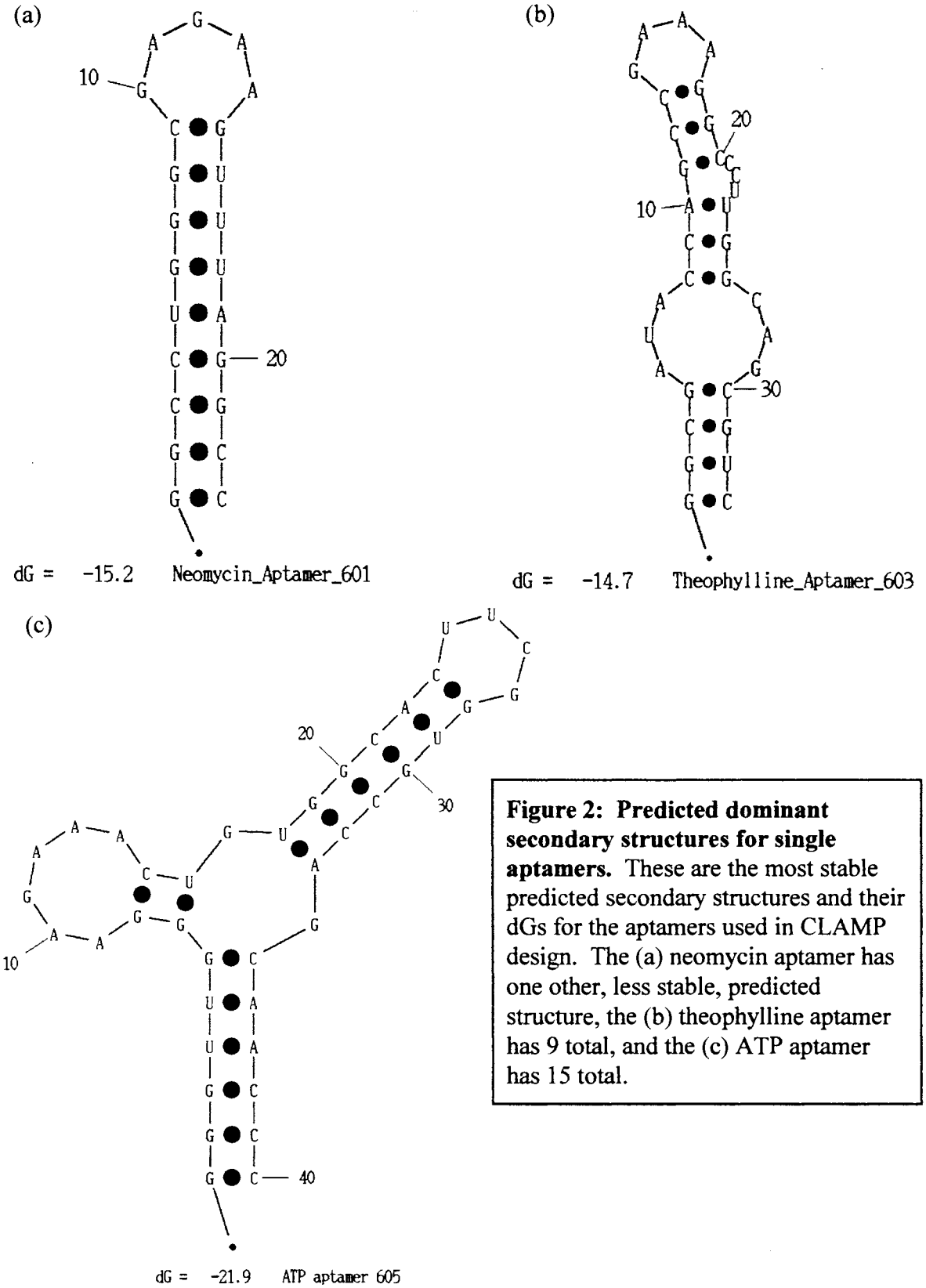


Figure 2: Predicted dominant secondary structures for single aptamers. These are the most stable predicted secondary structures and their dGs for the aptamers used in CLAMP design. The (a) neomycin aptamer has one other, less stable, predicted structure, the (b) theophylline aptamer has 9 total, and the (c) ATP aptamer has 15 total.

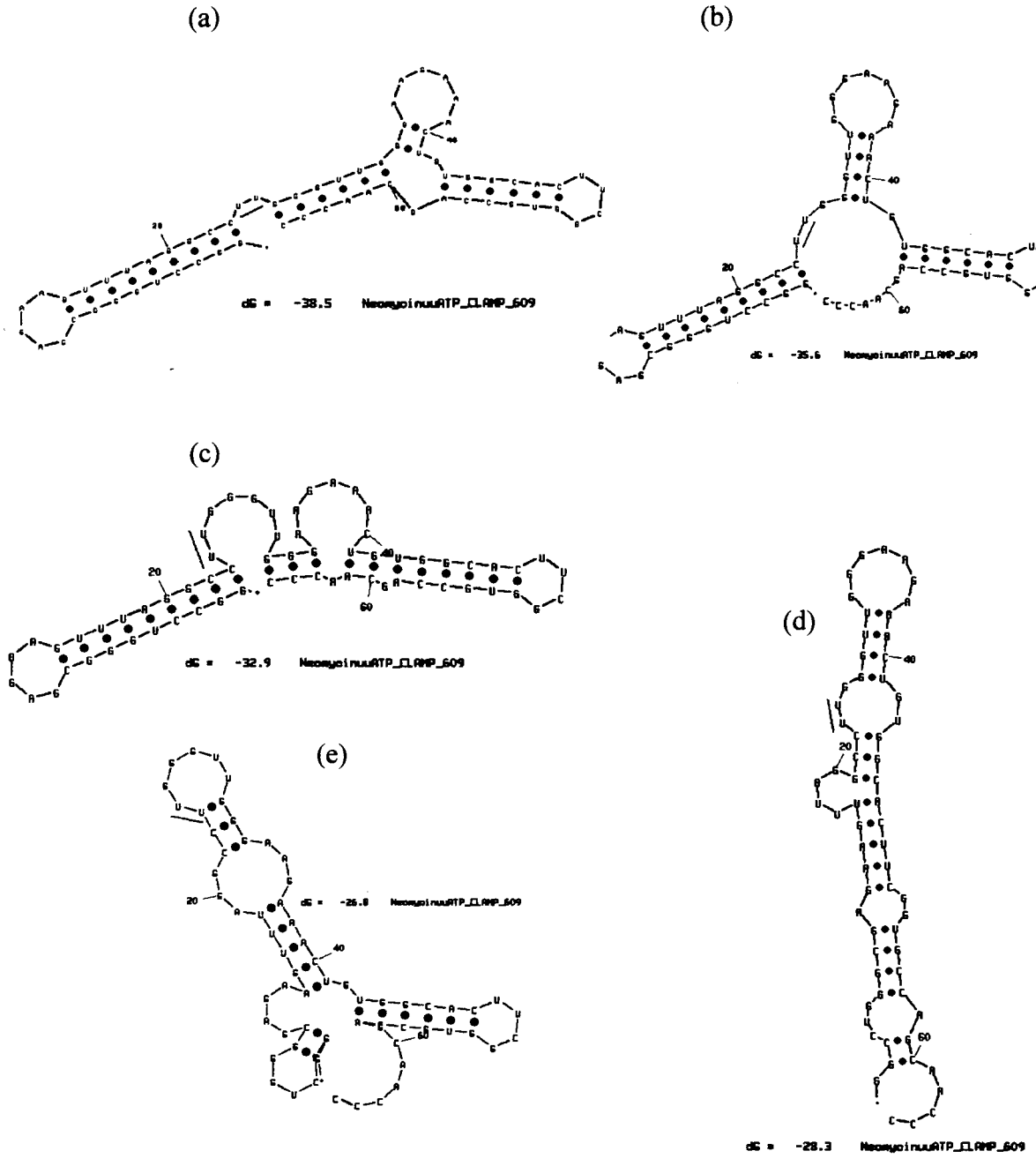
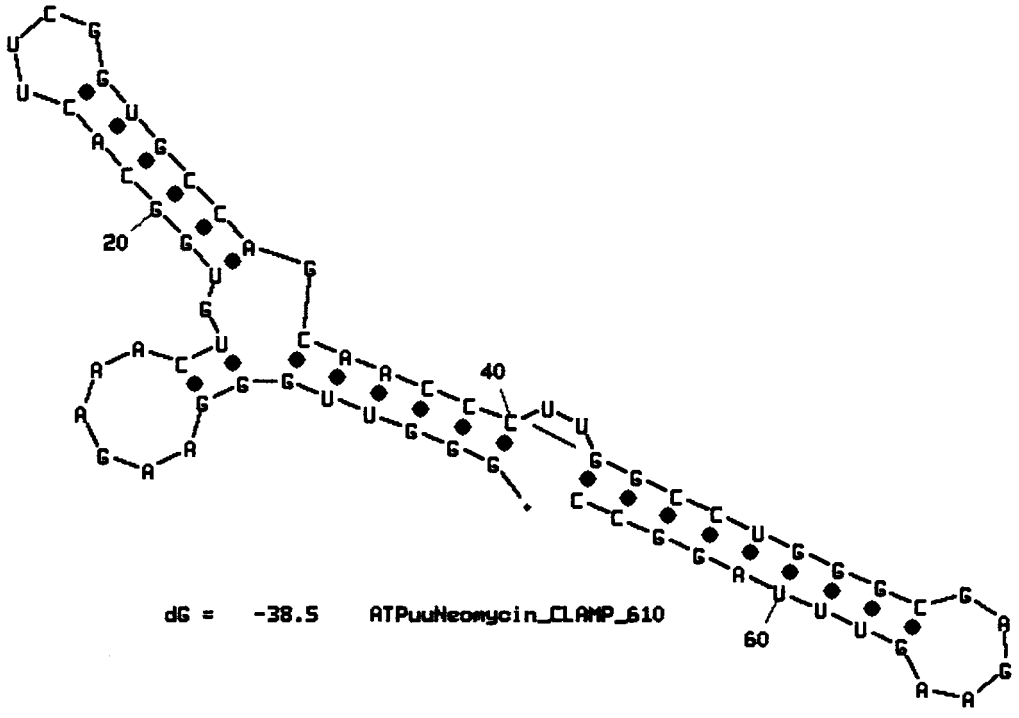


Figure 3: Multiple possible secondary structures of Neomycin-uu-ATP CLAMP 609. There are 15 secondary structures predicted for this CLAMP. The 5 structures and dGs shown are the (a) first and (b) second most stable structures and (c) - (e) a sampling of the other predicted secondary structures. Linkers are underlined.

(a)



(b)

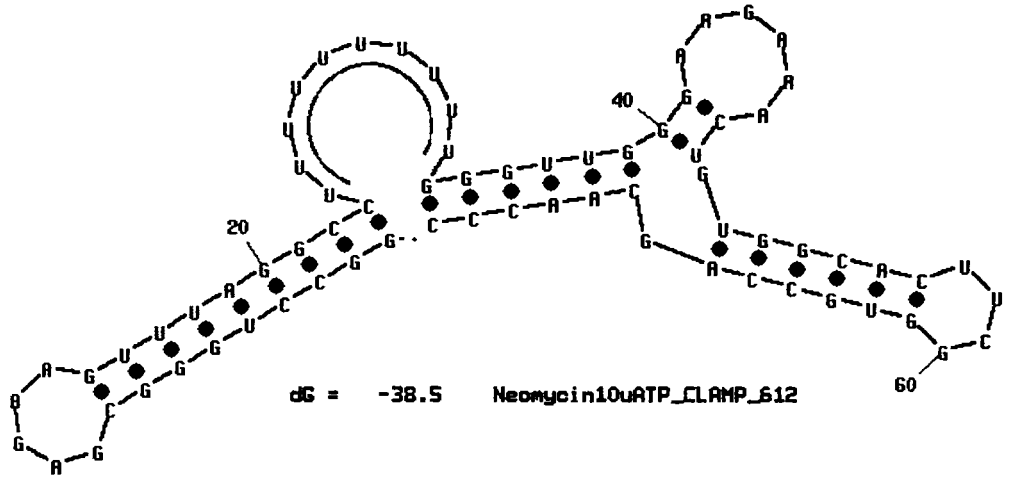


Figure 4: The most stable predicted secondary structure of (a) ATP-uu-Neomycin CLAMP 610 and (b) Neomycin-10u-ATP CLAMP 612. The most stable structure and its dG is shown out of 19 predicted for ATP-uu-Neomycin CLAMP 610 and over 20 predicted for Neomycin-10u-ATP CLAMP 612. Linkers are underlined.

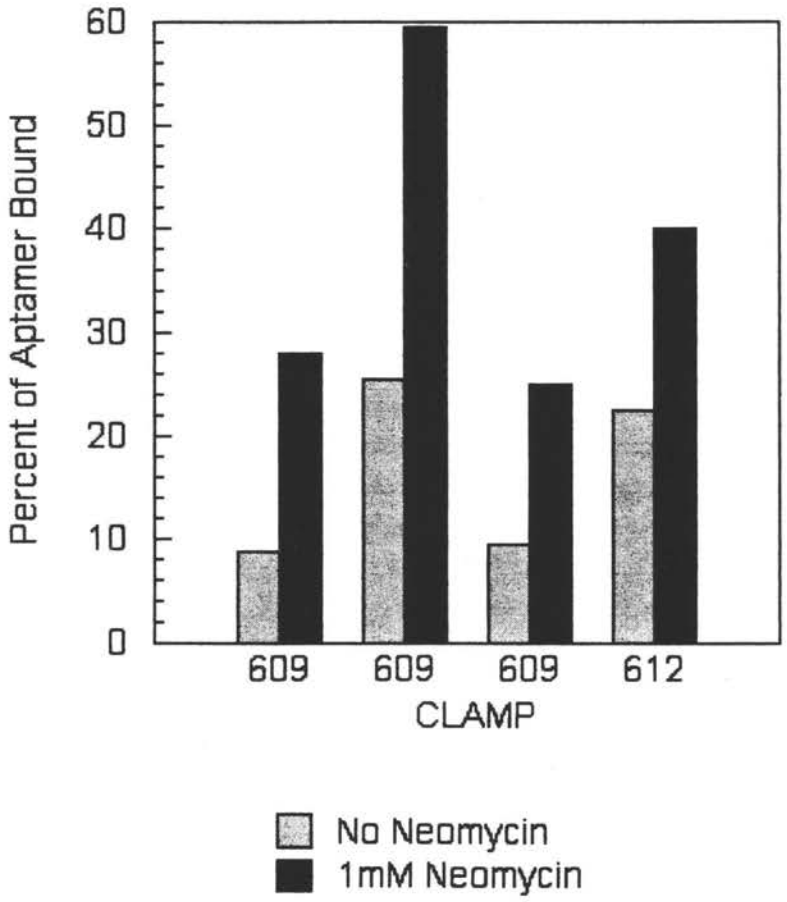


Figure 5: Neomycin increases ATP binding of Neomycin/ATP CLAMPs. The addition of 1mM neomycin to affinity column binding conditions approximately doubles the ATP binding of Neomycin-uu-ATP CLAMP 609 and Neomycin-10u-ATP CLAMP 612 (177%-323% of original ATP binding seen when neomycin is added). Separate trials are shown.

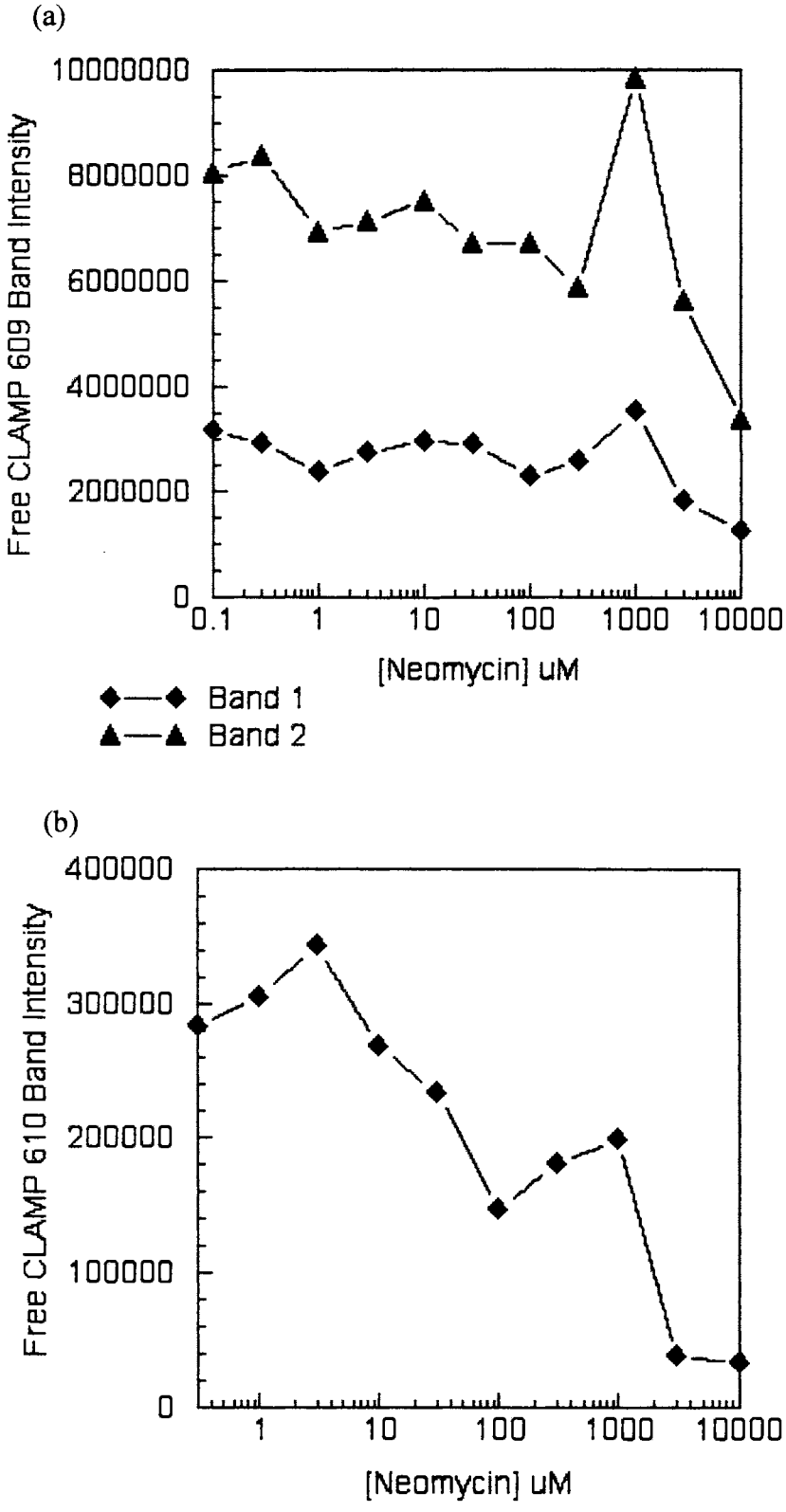


Figure 6: Neomycin increases ATP binding of Neomycin/ATP CLAMPs in gel mobility shift assays. A range of neomycin concentrations were added to 10 pmol RNA samples of (a) Neomycin-uu-ATP CLAMP 609 or (b) ATP-uu-Neomycin CLAMP 610. ATP agarose was added to retain ATP-bound CLAMP in the wells, and the samples were run on native acrylamide gels. Bands of CLAMP not bound to the ATP agarose were quantified and compared. (a) is representative of two separate experiments.

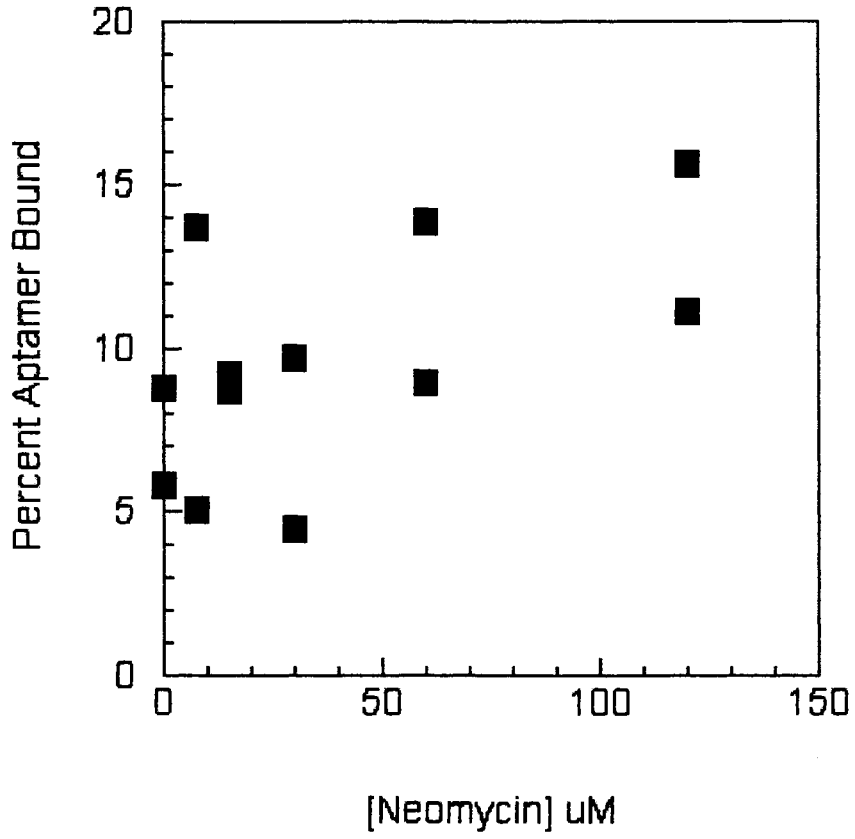


Figure 7: Neomycin increases ATP binding of Neomycin-10u-ATP CLAMP 612 in solution by equilibrium dialysis. CLAMP 612 at 13.4 uM was incubated for 22 hours with 0-120 uM neomycin and α^{32}P-ATP. The distribution of α^{32}P-ATP was measured between the RNA and buffer chambers, and the binding of the CLAMP determined from this difference.

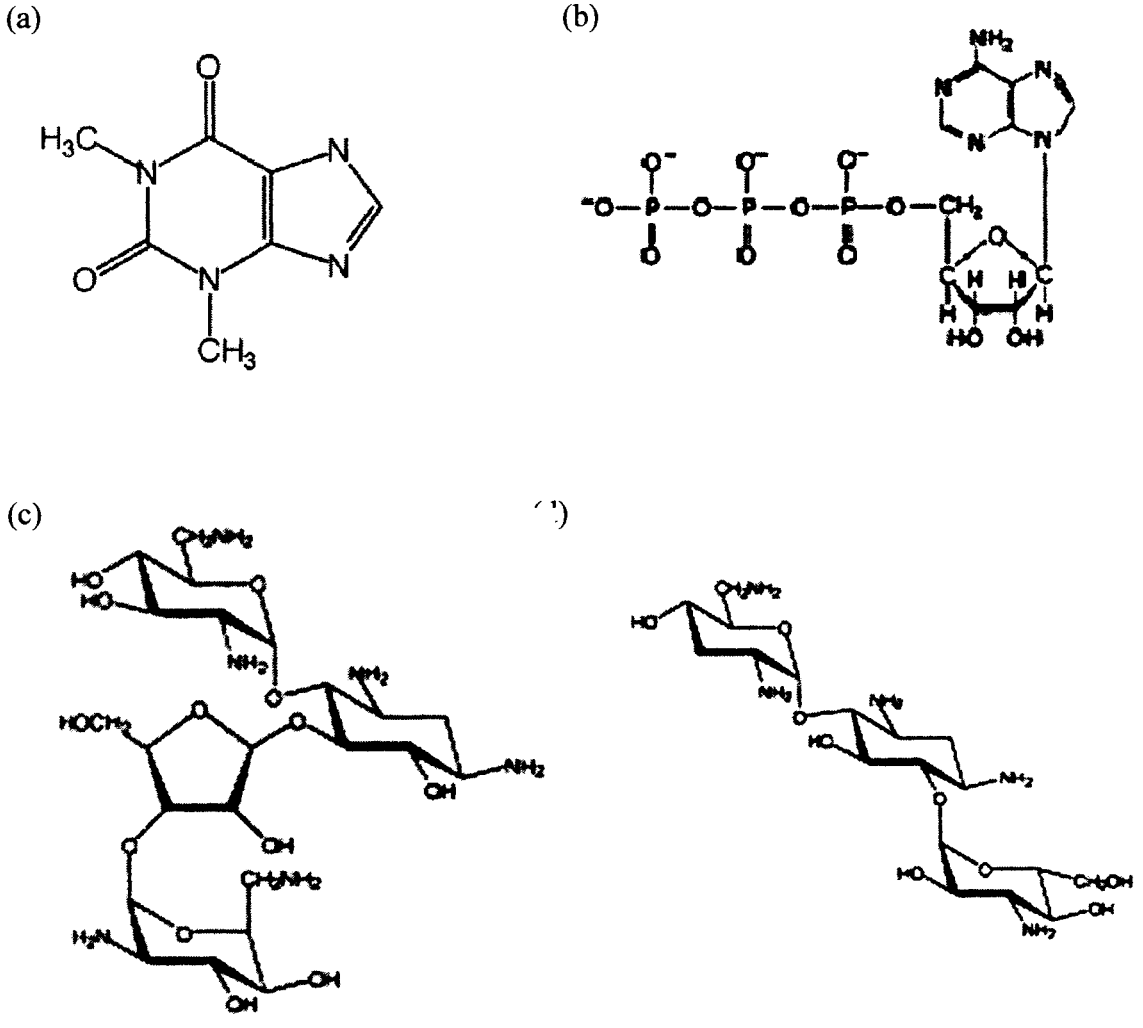
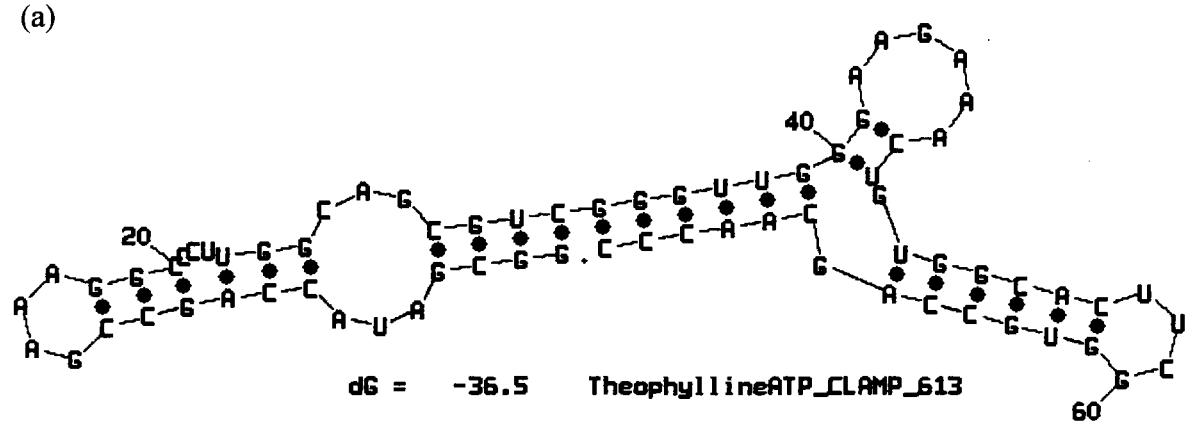


Figure 8: Theophylline, ATP, and aminoglycoside structures. Structures of the CLAMP ligands (a) theophylline and (b) adenosine 5'-triphosphate (ATP), and aminoglycosides (c) neomycin B and (d) tobramycin are shown.

(a)



(b)

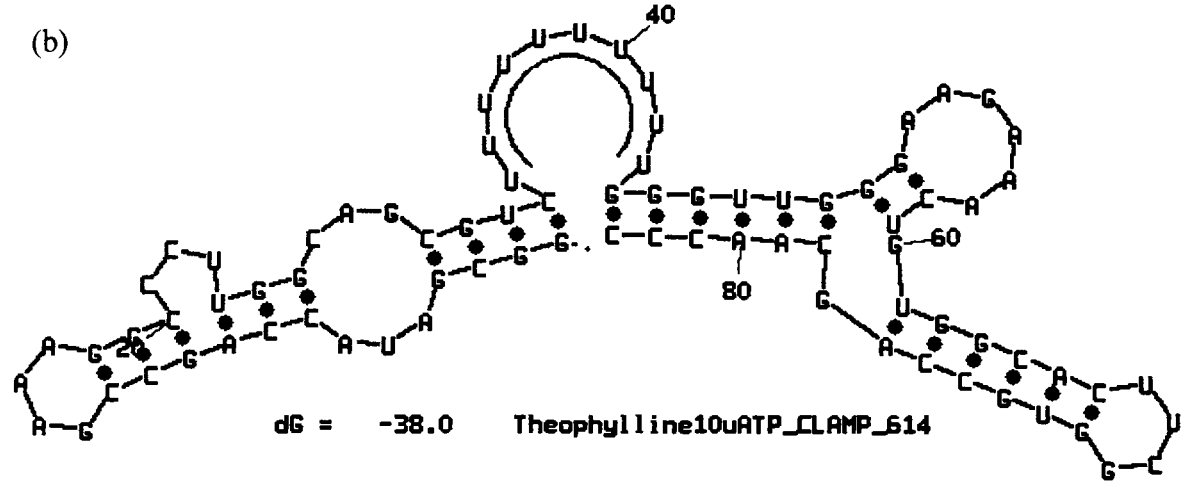


Figure 9: The most stable predicted secondary structures of (a) Theophylline-ATP CLAMP 613 and (b) Theophylline-10u-ATP CLAMP 614. The most stable structure and its dG is shown out of over 20 predicted for each CLAMP. The linker in (b) is underlined.

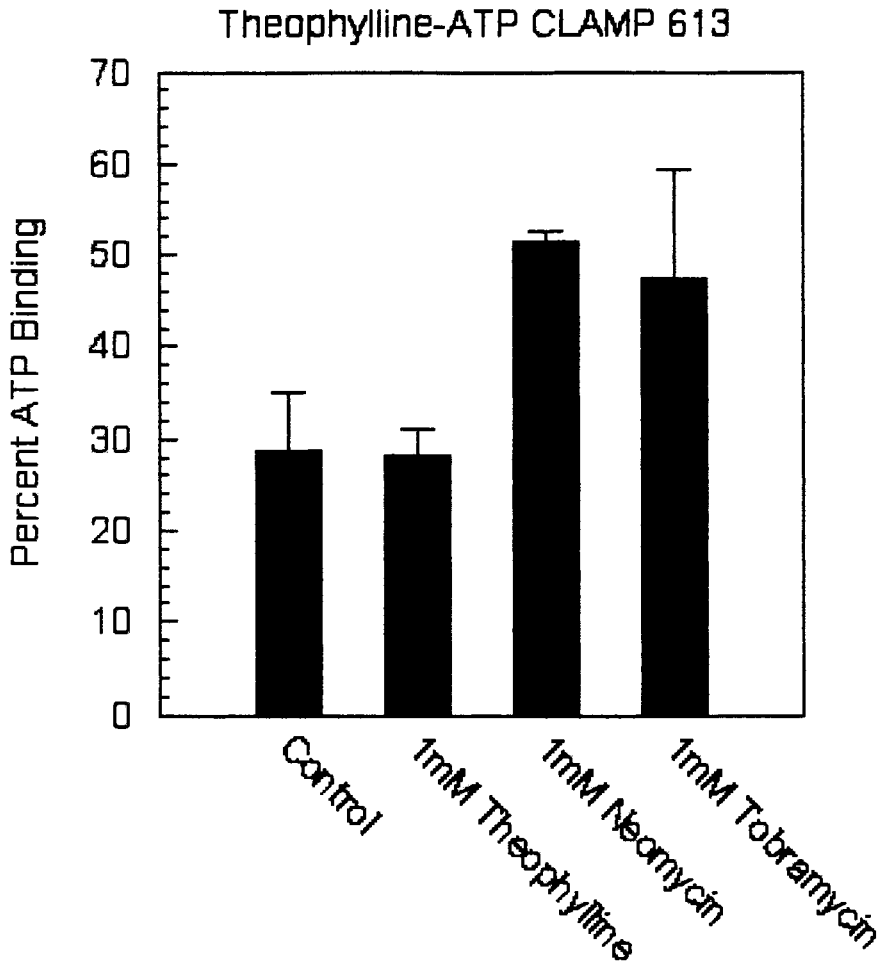


Figure 10: Aminoglycosides, but not theophylline, increase ATP binding of Theophylline-ATP CLAMP 613. Neomycin or tobramycin at 1mM increase the ATP binding ability of Theophylline-ATP CLAMP 613, with no linker between the aptamers (neomycin: $p < 0.0005$; tobramycin: $p < 0.05$). Theophylline does not influence the ATP binding of this CLAMP ($p = 0.85$). Affinity column binding data is represented, n=6 for control, n=4 for other conditions.

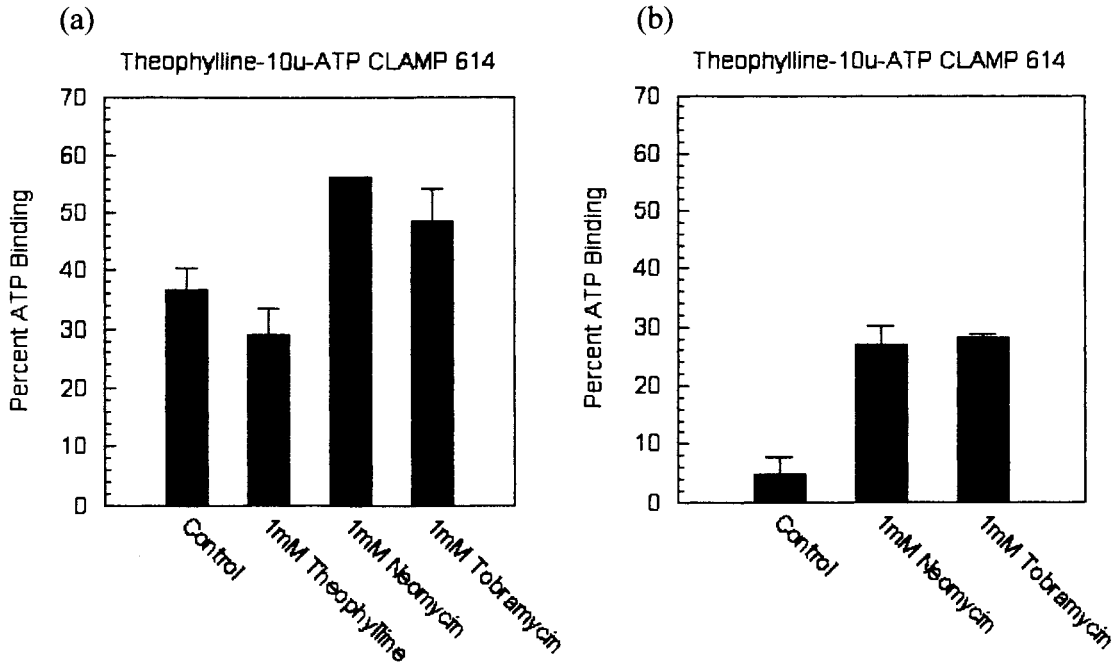


Figure 11: Aminoglycosides increase ATP binding of Theophylline-10u-ATP CLAMP 614, and theophylline may decrease ATP binding slightly. (a) Neomycin or tobramycin at 1mM increase the ATP binding ability of Theophylline-10u-ATP CLAMP 614, with a 10 uracil linker between the aptamers (not significant for this set of data, tobramycin: $p = 0.21$). Theophylline shows a slightly significant reduction in ATP binding ($p = 0.03$). (b) With a different RNA preparation, overall ATP binding is lower, but neomycin and tobramycin also increase ATP binding ability (neomycin: $p < 0.02$, tobramycin: $p < 0.07$). Affinity column binding data is represented, (a) n=6 for control, n=4 for theophylline, n=1 for neomycin, and n=2 for tobramycin, (b) n=2 for control, n=6 for neomycin, and n=2 for tobramycin.

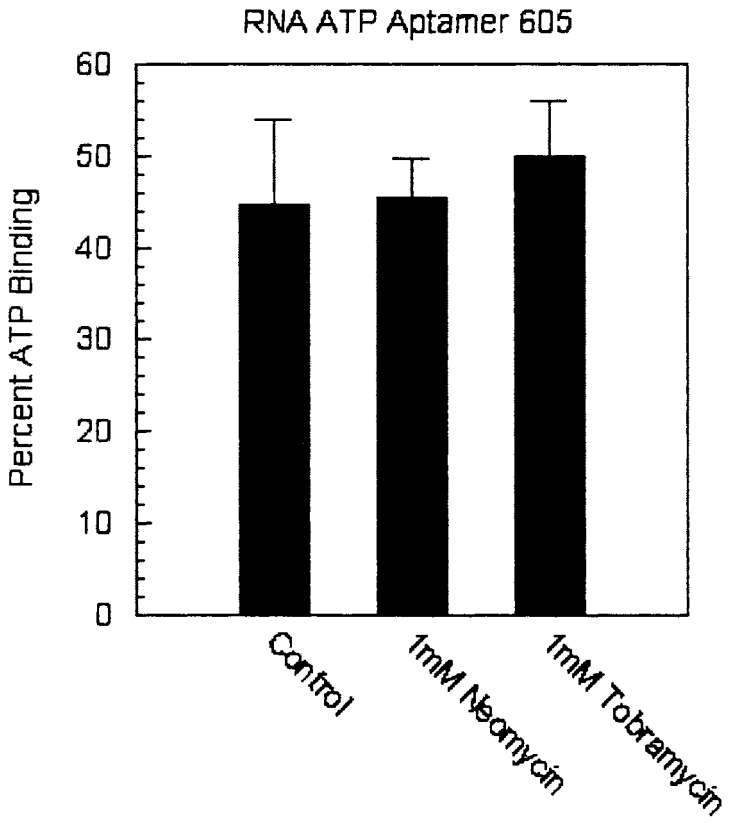


Figure 12: Aminoglycosides do not increase ATP binding of the RNA ATP aptamer 605 alone. Binding of the ATP aptamer used in the Theophylline/ATP CLAMPs 613 & 614 is not affected by 1mM neomycin or tobramycin (neomycin: $p = 0.88$, tobramycin: $p = 0.39$). Affinity column binding data is represented, n=4 for all conditions.

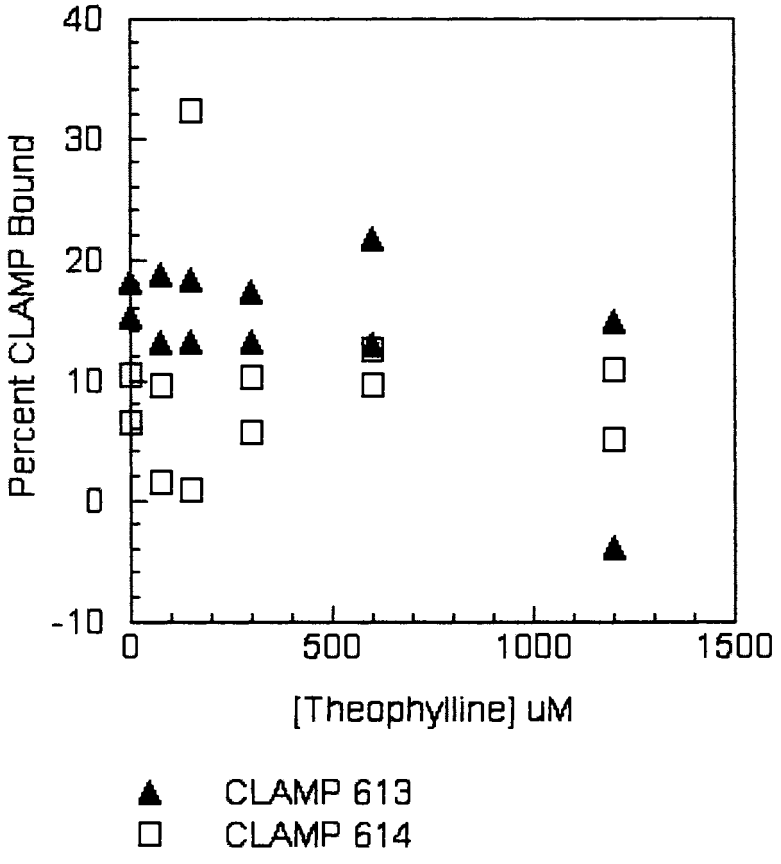


Figure 13: Theophylline does not affect ATP binding of Theophylline/ATP CLAMPs in solution as measured by equilibrium dialysis. Theophylline-ATP CLAMP 613 or Theophylline-10u-ATP CLAMP 614 at 15uM was incubated for 22 hours with 0-1.2mM theophylline and α^{32}P-ATP. The distribution of α^{32}P-ATP was measured between the RNA and buffer chambers, and the binding of the CLAMP determined. Results shown are representative of two separate experiments.

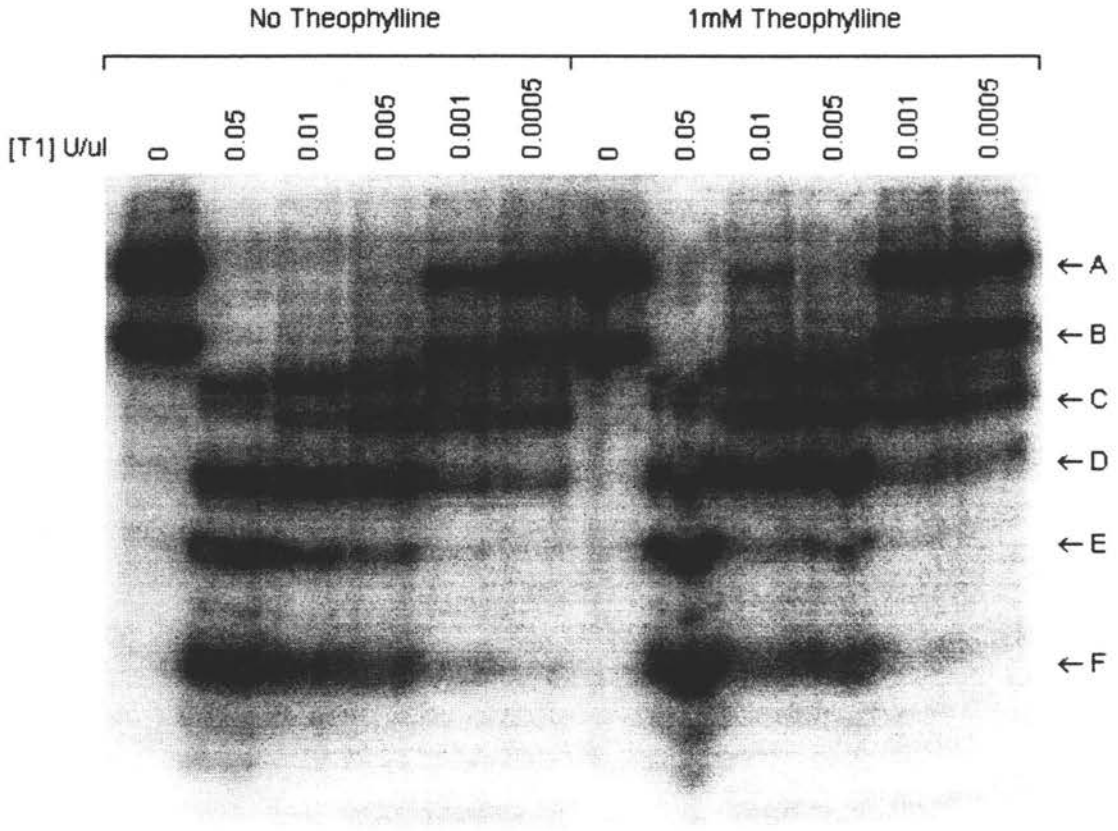


Figure 14: Theophylline-ATP CLAMP 613 is partially protected from T_1 cleavage by theophylline. Various concentrations of ribonuclease-T_1 were incubated for 5 minutes with 50pmol (5uM) of uniformly ^{32}P-labeled Theophylline-ATP CLAMP 613, with or without 1mM theophylline. The samples were then immediately run on a 20% native polyacrylamide gel. The bands labeled A-F were quantified (see Table 2) in the 0.01 and 0.001 U/ul samples and compared to see the protection caused by theophylline binding.

Table 2: Quantified Bands from T_1 Cleavage Protection in Figure 12.

No Theophylline			1mM Theophylline			Percent of original intensity upon Theophylline addition
$[T_1]$	Band	Intensity	$[T_1]$	Band	Intensity	
0.01	A	9.00E+06	0.01	A	4.49E+07	499
0.01	B	2.93E+07	0.01	B	1.30E+08	444
0.01	C	7.94E+07	0.01	C	2.80E+08	353
0.01	D	3.34E+08	0.01	D	1.83E+08	55
0.01	E	1.33E+08	0.01	E	2.65E+07	20
0.01	F	1.27E+08	0.01	F	5.32E+07	42
0.001	A	1.43E+08	0.001	A	3.02E+08	212
0.001	B	1.85E+08	0.001	B	3.16E+08	171

Conclusion

The ability of aminoglycosides to bind to RNA constructs containing aptamer sequences and influence the aptamer activity provides great opportunities for the rational design of allosteric aptamer constructs. As aminoglycoside binding motifs become more clearly recognizable, it will be easier to attach a binding sequence to the desired aptamer, potentially creating an aptamer with modifiable binding to its ligand without requiring an allosteric selection. Tunable aptamers could be very useful as therapeutic or diagnostic molecules, with primary ligand binding under control of a separate ligand. Aminoglycosides may even act as chaperones for RNA folding, opening up new applications for aminoglycosides when combined with RNA therapies or detection assays.

However, the fact that the aminoglycosides can affect the activity of aptamer constructs intended for binding totally different ligands suggests possible problems in using aminoglycosides in aptamer applications. There may be effects from selective binding of the aminoglycosides when only specific aptamer-ligand binding was desired. Overall, the wide range of applications for using aminoglycosides to affect aptamer binding should compensate for the difficulty in controlling the selective binding effects of these versatile molecules.

CHAPTER 3: APTAMER LINKING OF FLUORESCENT SILICA NANOSPHERES

Abstract

Nucleic acid aptamers have the potential to become a valuable tool for numerous biotechnological applications. Aptamers can rival antibodies for specificity and affinity to their target ligand, yet are much smaller and more easily manipulated for various applications *in vitro* or *in vivo*. Aptamers are ideal for use in combination with mesoporous silica nanospheres, which can be designed with multiple fluorophores contained within and with various ligands attached covalently to the outside surface or the pore surfaces within the spheres. In this manner dual aptamer constructs, also called Cis-Linked Aptamers for Microanalytical Procedures (CLAMPs), can be used to bind two species of fluorescent silica nanospheres together. When filled with appropriate donor and acceptor fluorophores, the two species of nanospheres can produce a new fluorescent signal by Fluorescence Resonance Energy Transfer (FRET) when bound close together by the CLAMPs. In this way logic gates such as "AND", "OR", and "NOR" can be constructed, relying upon the signal from this system of aptamers and nanospheres.

Introduction

Mesoporous silica nanospheres (MSN) provide an exciting new medium for testing the limits of aptamer technology. Forming spherical particles 200 nm in diameter, these nanospheres have 2.3 nm diameter cylindrical pores arranged hexagonally that pass entirely through the nanospheres (*56*). The cylindrical, hexagonally arranged pores give the

mesoporous silica nanospheres a designation of MCM-41 type (Figure 15). These channels are formed during production of the nanospheres by the co-condensation method (*57, 58*) developed by Victor Lin's group. The nanospheres can be separately derivatized via several different chemical linking groups (Figure 16), linking ligands for the aptamers to the surface of the nanospheres or the inside surfaces of the pores. Various fluorophores can be contained inside the nanosphere material to create the different fluorescent nanospheres required for producing fluorescence resonance energy transfer (FRET).

An "AND" logic gate system, designed by Victor Lin, uses different species of nanospheres as the inputs, with Cis-Linked Aptamers for Microanalytical Procedures (CLAMPs) as the mechanism for linking the nanospheres together to create the FRET output (Table 3). One aptamer ligand is derivatized to the surface of nanospheres containing Texas Red, the energy acceptor, and a second aptamer ligand is derivatized to the surface of CdS particles, the energy donor. When a CLAMP, containing both aptamer sequences, binds to both of these ligand derivatized nanoparticles, the Texas Red and CdS are brought close enough together for FRET to occur, resulting in a new fluorescent emission which can be measured as the positive output of this system. In this way, logic gates can be constructed that function as an optical version of the standard electronic data storage device.

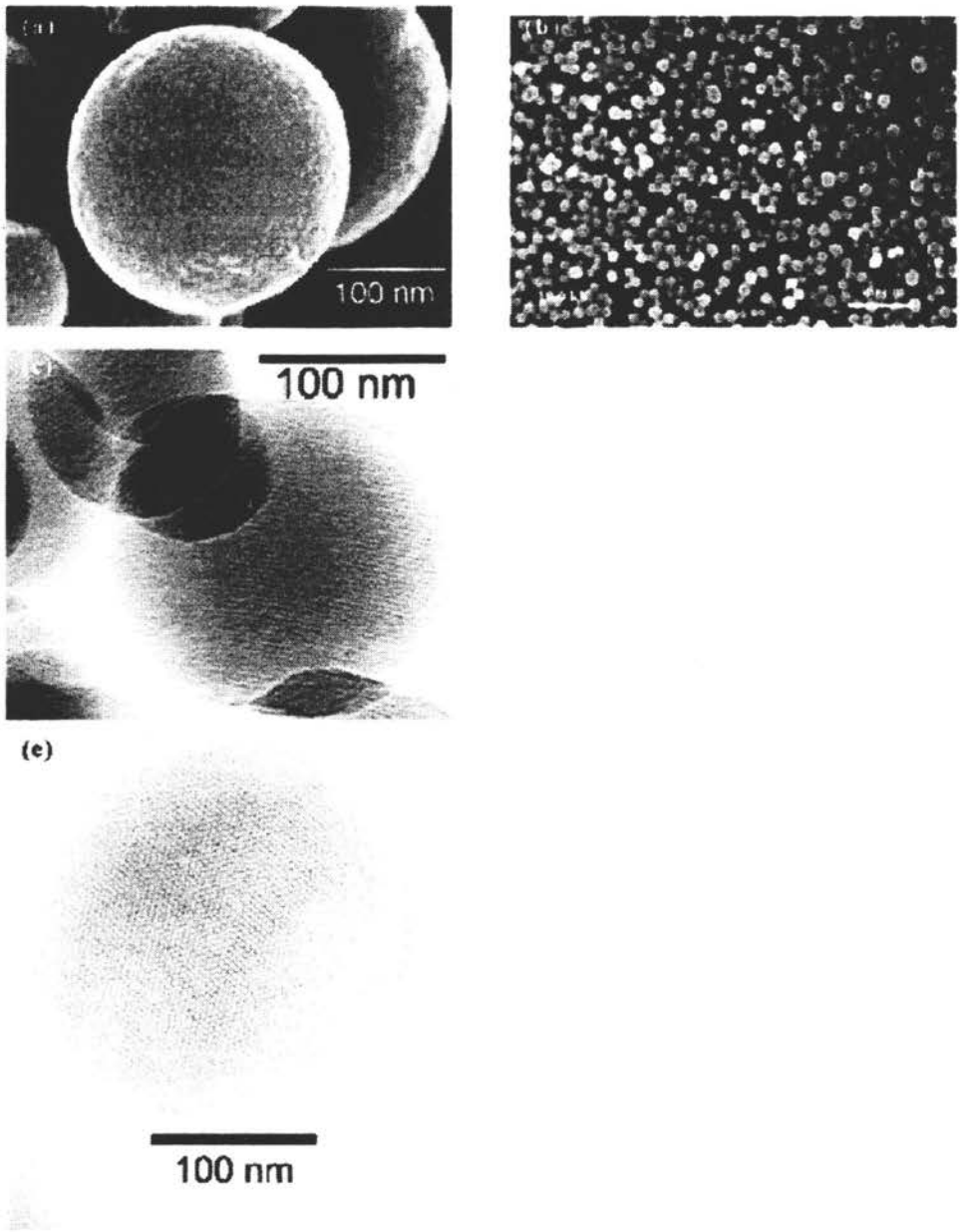


Figure 15: SEM (a and b) and TEM (300kV) micrographs of the mesoporous silica nanospheres (c and e). The MCM-41 type of mesoporous channel structure of the nanospheres is visualized with the parallel stripes (c) and the hexagonally packed light dots (e) shown in the micrographs. The TEM micrograph (e) was measured on ultramicrotomed samples with section thickness of 60-80 nm. This figure is modified from (*56*), with panels d & f removed.

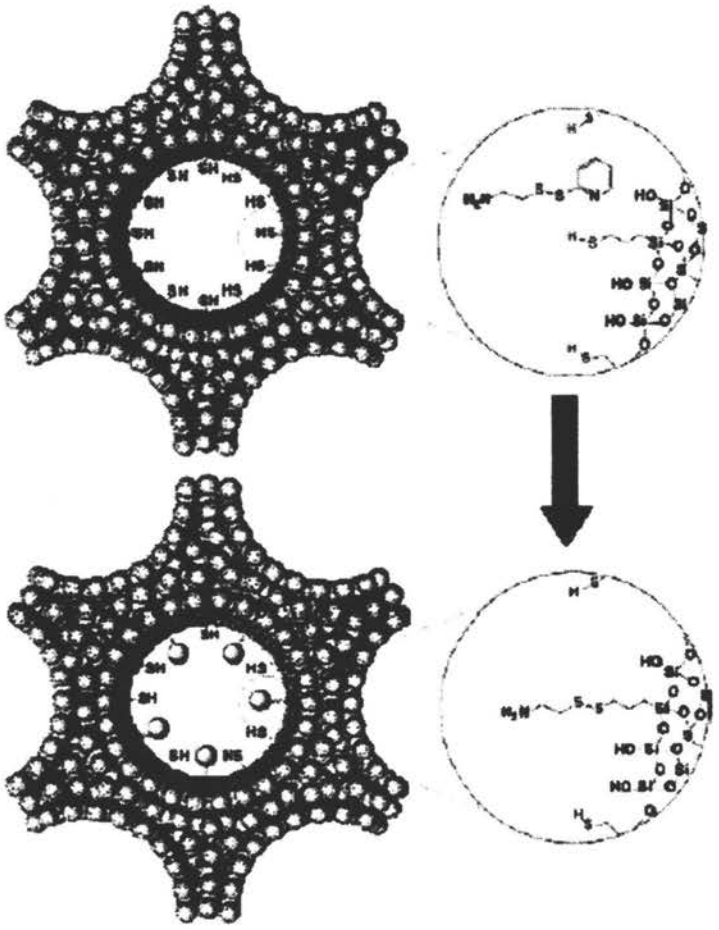


Figure 16: Synthesis of 2-(propyldisulfanyl)ethylamine functionalized mesoporous silica nanosphere material. This figure is taken from (*56*).

Table 3: AND Logic Gate System Design*

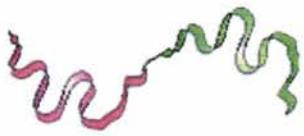
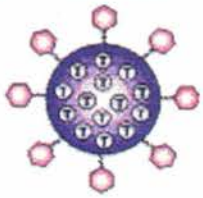
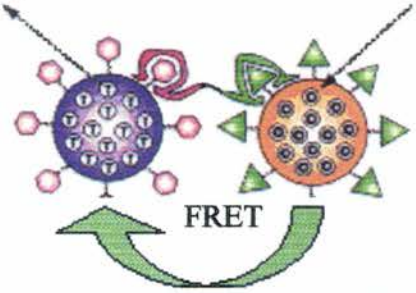
	AND Logic Gate System	
		**CLAMP #1: Selectively bind to neomycin B and adenosine**
Input 1		**Nanosphere (A): CdS inside and neomycin B outside**
Input 2		**Nanosphere (B): Texas Red inside and adenosine outside**
Output	output *hv* *hv* 	**FRET from CdS to Texas Red gives rise to new emission as signal output**

*table and figures from Victor Lin

In addition to the specified goal of creating a logic gate system which may be applicable as an optical switch or for data storage technology, being able to link particles together using aptamer constructs would be useful for other applications involving aptamers. For instance, therapeutic molecules could be targeted to a cell type expressing a specific cell surface antigen for targeted drug delivery.

Materials and Methods

Neomycin and adenosine were purchased from Sigma. Tris base, sodium chloride, and magnesium chloride were purchased from Fisher Scientific.

Nanosphere and nanoparticle synthesis. Mesoporous silica nanospheres were prepared by the co-condensation method (*57, 58*) by Daniela R. Radu. Texas Red nanospheres were derivatized with adenosine via an amino linkage to the C8 position of the adenosine molecule, as required for aptamer binding. CdS particles were linked to neomycin also via an amino linkage.

RNA CLAMP preparation. See Chapter 2, Materials and Methods.

Column affinity CLAMP binding assay. See Chapter 2, Materials and Methods.

FRET experiments. Sonication was often used to disperse the nanospheres and CdS particles in solution but because this may shear the nucleic acid CLAMPs, sonication was only used before the addition of CLAMP. Fluorescence emission spectra were measured on a Fluoro Max-2 Fluorometer (ISA Jobin Yvon Spex, Horiba Group). The experimental order was as follows: emission spectrum of Texas Red nanospheres and CdS nanoparticles in buffer was measured, CLAMP sample was added, and the emission spectrum was measured again,

unless otherwise noted. The buffer for all FRET experiments was 50 mM Tris, 250 mM NaCl, 5 mM $MgCl_2$, pH 7.6 at 23°C.

Results and Discussion

Three CLAMPs were designed combining the neomycin and ATP RNA aptamers with varying linkers (Table 1). Neomycin-uu-ATP CLAMP 609 has the neomycin aptamer 5' with two uracil nucleotides as a linker before the ATP aptamer at the 3' end. ATP-uu-Neomycin CLAMP 610 has the ATP aptamer 5' with a two uracil nucleotide linker and the neomycin aptamer at the 3' end. Neomycin-10u-ATP CLAMP 612 has the neomycin aptamer 5' with ten uracil nucleotides as a linker before the ATP aptamer at the 3' end.

The binding ability of both ends of the CLAMPs were first checked by affinity column chromatography. Neomycin-uu-ATP CLAMP 609 binds both neomycin and ATP independently, with a 10mM solution of the opposite ligand unable to elute the CLAMP from the affinity column (Figure 17). The amount of ATP binding obtained with this CLAMP was later increased from the low levels seen here (see Chapter 2). The independent binding abilities of the ATP-uu-Neomycin CLAMP 610 and the Neomycin-10u-ATP CLAMP 612 were similar when tested on ATP agarose and neomycin sepharose affinity columns. However, simultaneous binding of both ligands was not determined.

The emission spectra of the CLAMPs alone were checked with excitation at 360 nm to make sure they did not have a significant emission that would interfere with readings of the Texas Red emission. From the spectra of Neomycin-uu-ATP CLAMP 609 (Figure 18a) and ATP-uu-Neomycin CLAMP 610 (Figure 18b) there was very little emission when compared

with the intensity of CdS and Texas Red emissions, especially in the 600 nm range, where the peak of Texas Red emission is expected if FRET occurs. Therefore, there should be little interference in the Texas Red emission spectra from the nucleic acid CLAMPs themselves.

Changes in the Texas Red emission spectrum were first tested by measuring the spectrum of a solution of Texas Red nanospheres and either Neomycin-uu-ATP CLAMP 609 or ATP-uu-Neomycin CLAMP 610, then adding CdS particles and measuring the spectrum again (Figure 19). The addition of CdS particles caused large changes in the emission spectrum, increasing the CdS emission at 400-500 nm, and also decreasing the Texas Red emission peak at 600 nm. This made it difficult to determine whether any FRET occurred.

A better method for determining possible FRET is measuring the change in Texas Red emission with excitation at 360 nm between a solution of Texas Red nanospheres and CdS particles, before and after addition of CLAMP. The first results from this comparison are shown in Figure 20. The addition of Neomycin-uu-ATP CLAMP 609 (225 pmol) decreased emission evenly all across the spectrum. This was interpreted as a dilution effect from adding the 30 ul of CLAMP solution to the 2 ml sample. There appeared to be no FRET produced in this trial.

Another barrier to producing FRET may be balancing the amount of Texas Red nanospheres and CdS particles so there is enough CdS to transfer energy to the Texas Red. This balance was addressed in the next experiments by adjusting the relative amounts of Texas Red nanospheres and CdS particles until the emission peaks of each species at excitation 360 nm were of approximately equal intensity (Figure 21). Then Neomycin-10u-ATP CLAMP 612 was added in 3 increments and the emission spectra (excitation 360 nm) were compared

(Figure 22). Once again, the only change in the emission spectrum was a drop in the intensity of emissions caused by dilution when the CLAMP solution was added, with no evidence of FRET.

Spin column purification had been used to purify all RNA samples up to this point, and there was the possibility that this purification had impaired the binding ability of the CLAMPs. To test if this was the case, unpurified CLAMP samples were tested. The results (Figures 23 & 24) were somewhat misleading, somewhat resembling FRET, but actually resulting from several different effects. The decrease in CdS particle emission over the range 400-500 nm is not consistent with FRET to the Texas Red nanospheres because Texas Red should only absorb the higher end of this range, with the maximum absorption around 560 nm. Therefore, this decrease in CdS emission is probably just the dilution effect from addition of CLAMP solution. The increase in Texas Red emission at 600 nm is harder to explain but is believed to be due to increased solvation of the Texas Red nanospheres caused by electrostatic interactions between the silica nanospheres and the high concentrations of free nucleotide triphosphates present in the unpurified RNA samples. This may explain why the first 20 ul addition of Neomycin-10u-ATP CLAMP 612 was the only addition that increased the Texas Red emission (Figure 24, aqua line) with no further increases occurring in response to the remaining CLAMP additions. If the maximum amount of nucleotide triphosphates had been bound to the nanospheres with the first addition, adding more would not increase solvation (and therefore, emission) any further. If this increase in emission intensity was due to CLAMP binding, the amount of CLAMP should be limiting, and further additions should increase emission intensity further.

Purifying all further CLAMP samples appears to be important to avoid the previous problems. Upon further testing with purified CLAMP samples, more odd results were seen. Adding any of the three CLAMPs to a Texas Red nanosphere and CdS particle mixture caused a decrease in CdS emission from 400-500 nm as seen previously from the dilution effect, but higher wavelength emissions were increased (Figures 25-27). This is most easily seen in Figure 20, with Neomycin-uu-ATP CLAMP 609, where 525 nm is the start of the increased emission intensity. With Neomycin-10u-ATP CLAMP 612, increased emission intensity begins at about 480 nm (Figure 27). This increase in primarily Texas Red emission may be due to increased solvation like that seen with the unpurified CLAMP samples, caused by CLAMP molecules binding to the Texas Red nanospheres and neutralizing some of the silica material's hydrophobicity. In these final trials, still no FRET was observed.

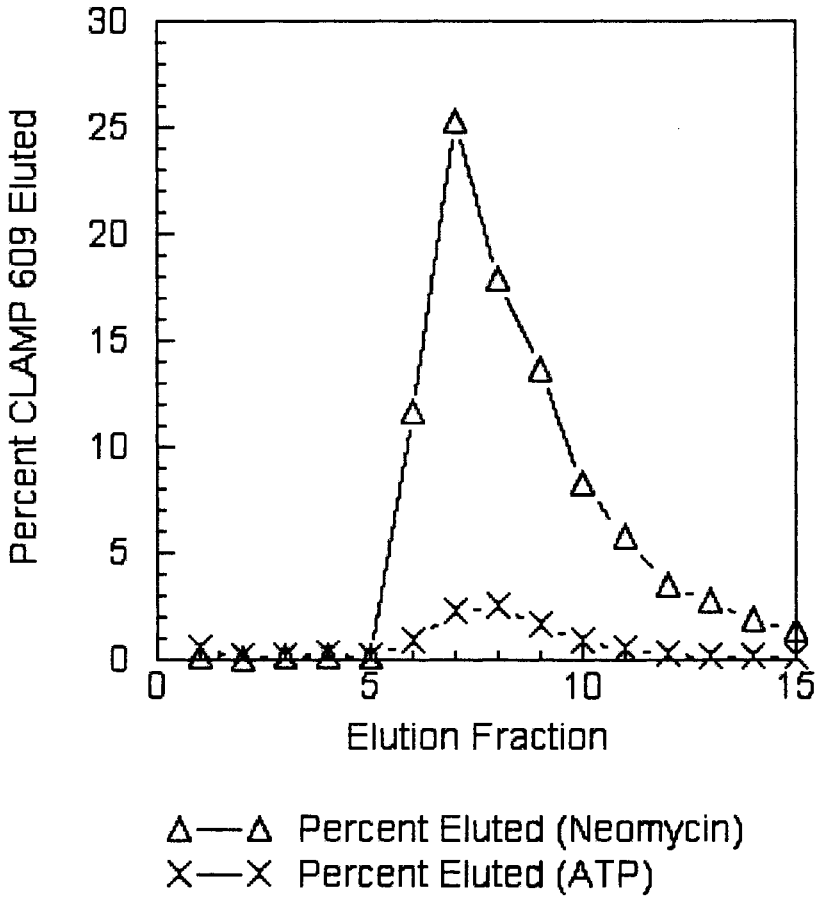


Figure 17: Independent binding of neomycin and ATP by Neomycin-uu-ATP CLAMP 609. CLAMP was allowed to bind to either a neomycin or ATP affinity column, washed as normal, then washed with 5 column volumes of a 10mM solution of the opposite ligand (elution fractions 1-5) before elution with a 10mM solution of the proper ligand (elution fractions 6-15). Representative affinity column binding data from two separate trials is shown.

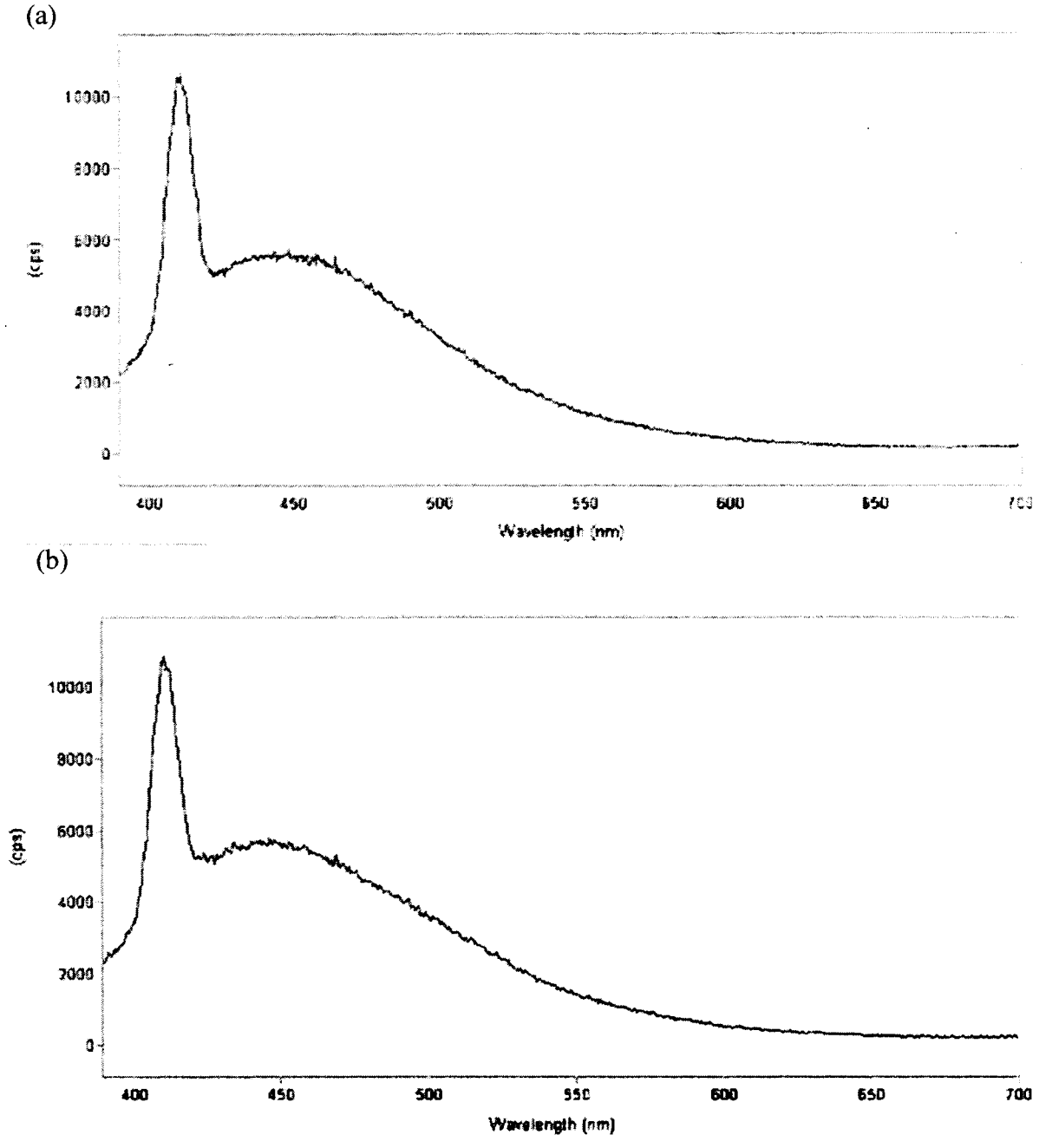
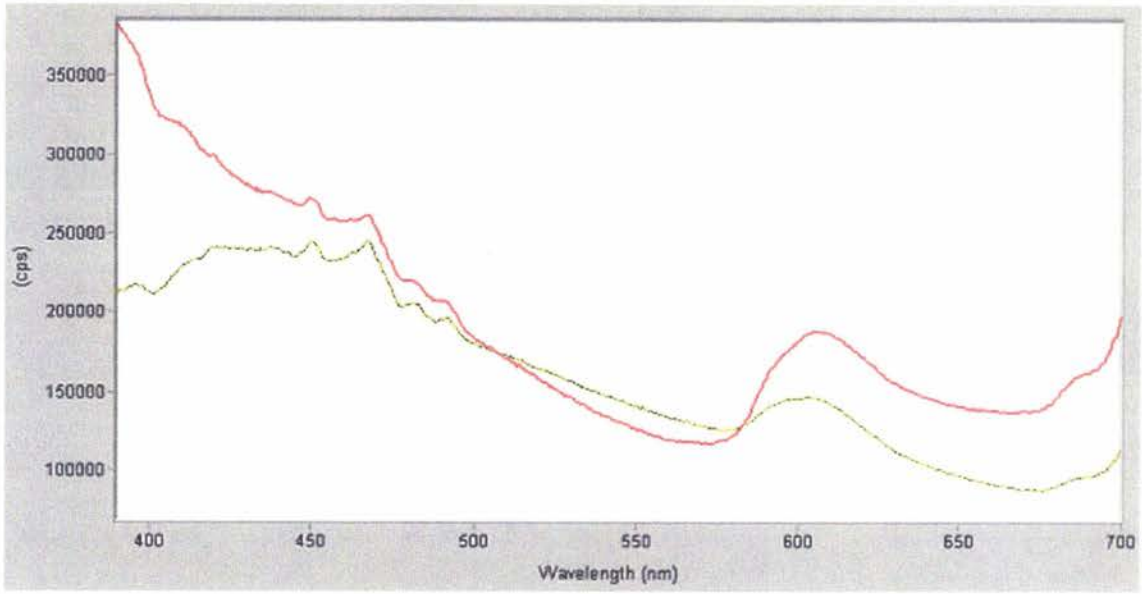


Figure 18: Emission spectra of Neomycin-uu-ATP CLAMP 609 and ATP-uu-Neomycin CLAMP 610. With excitation at 360 nm, the wavelength for exciting CdS particles, (a) Neomycin-uu-ATP CLAMP 609 and (b) ATP-uu-Neomycin CLAMP 610 have very low emission at the Texas Red emission peak of 600 nm.

(a)



(b)

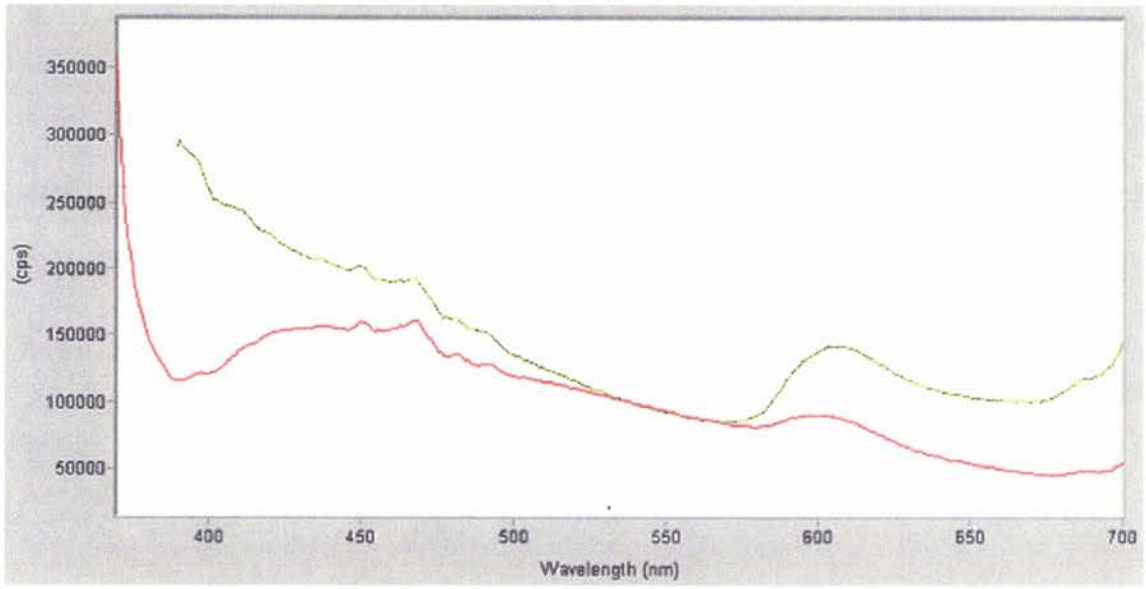


Figure 19: Emission spectra of Neomycin-uu-ATP CLAMP 609 or ATP-uu-Neomycin CLAMP 610 and Adenosine derivatized Texas Red Nanospheres with and without Neomycin derivatized CdS particles. Texas Red nanospheres ((a) 2.1 mg, (b) 2.3 mg) were sonicated in 2 ml aptamer binding buffer, then (a) Neomycin-uu-ATP CLAMP 609 or (b) ATP-uu-Neomycin CLAMP 610 was added and emission spectrum was measured with excitation at 360 nm ((a) red, (b) green). CdS particles ((a) 1.9 mg, (b) 2.4 mg) were added and the mixture sonicated before measuring new emission spectrum ((a) green, (b) red).

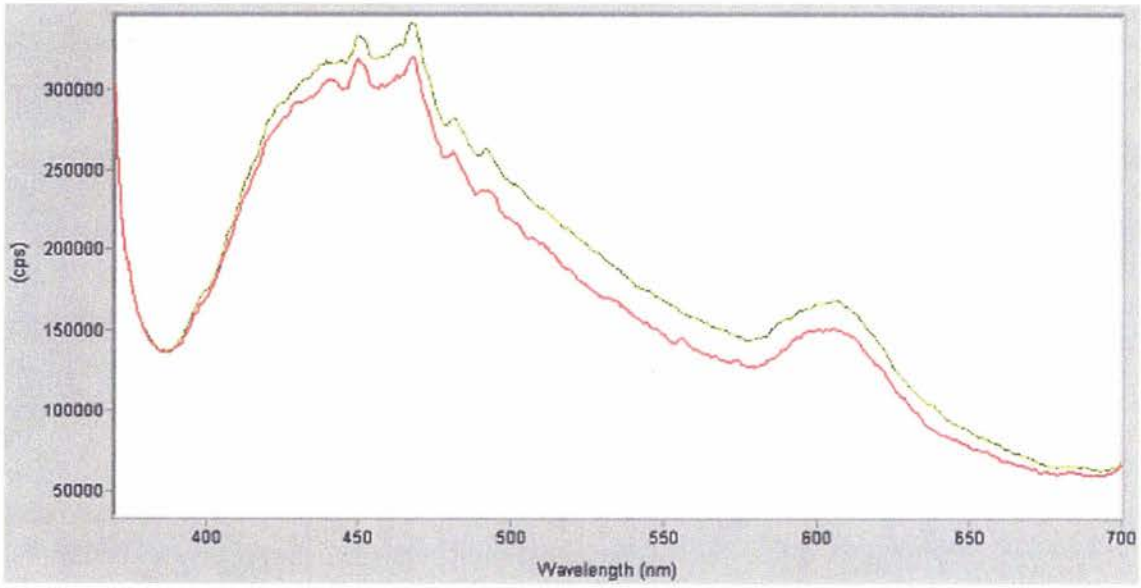


Figure 20: Emission spectrum of Texas Red Nanospheres and CdS particles with and without Neomycin-uu-ATP CLAMP 609. Adenosine derivatized Texas Red nanospheres (5.4 mg) were mixed with neomycin derivatized CdS particles (4.4 mg) in 2 ml of aptamer binding buffer and sonicated to disperse. Emission spectrum was measured at excitation 360 nm (green), then Neomycin-uu-ATP CLAMP 609 (225 pmol in 30ul) was added and emission spectrum measured again (red).

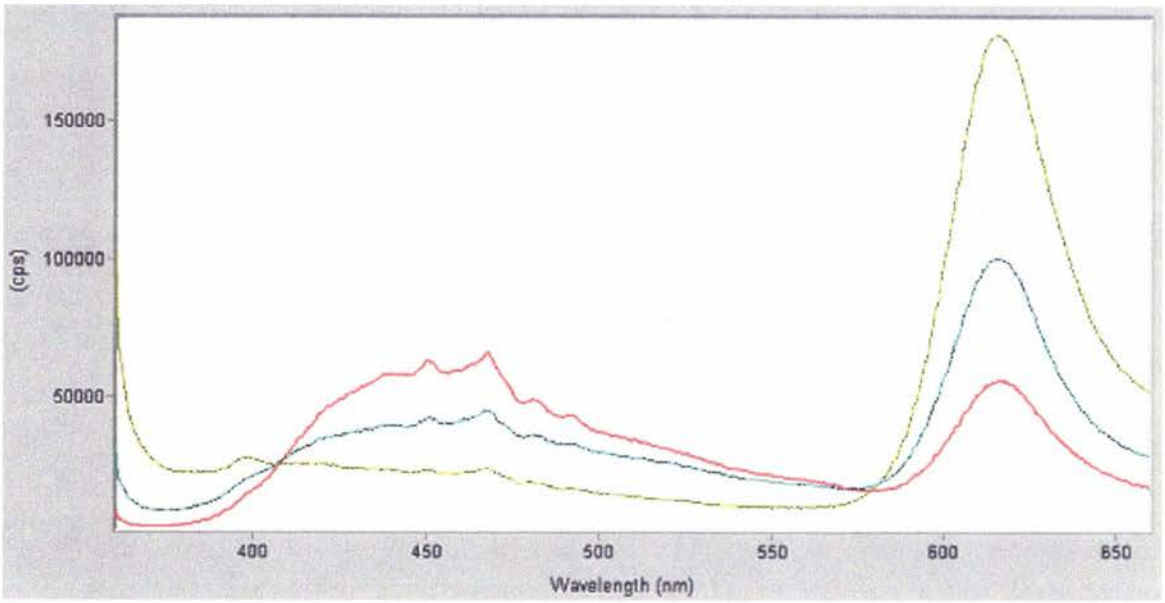


Figure 21: Balancing the CdS and Texas Red emission peaks. The relative amounts of CdS particles and Texas Red nanospheres were adjusted (green → blue → red) until the two emission peaks at excitation 360 nm were about equal (red), with 4.5 mg CdS particles and 1.5 mg Texas Red nanospheres in 2 ml of aptamer binding buffer.

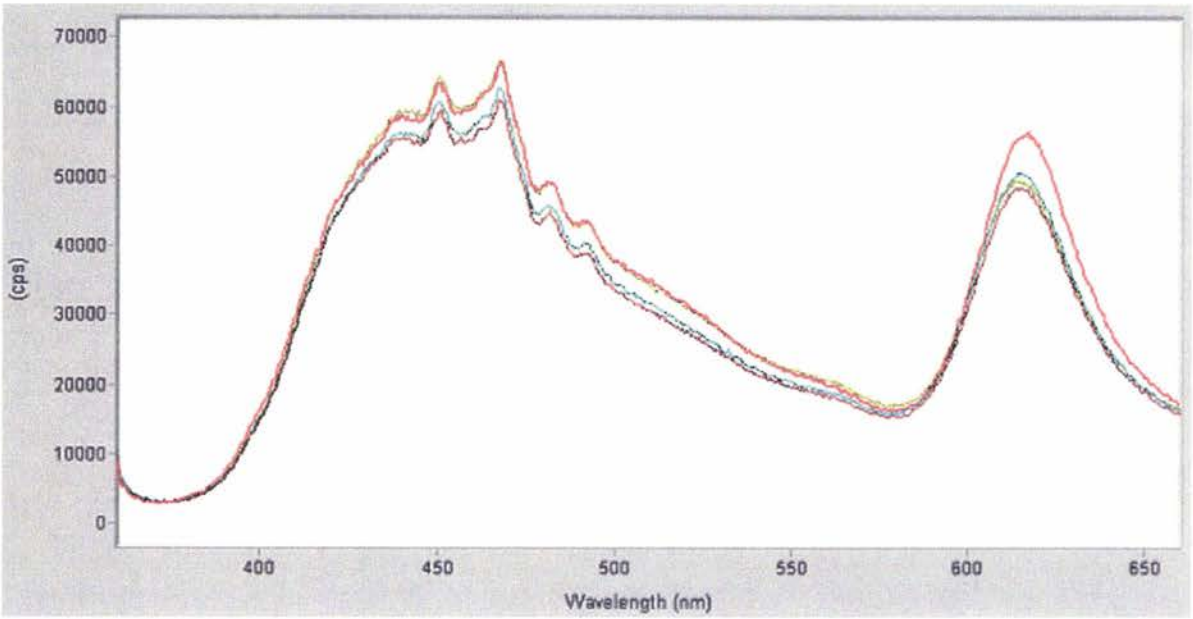
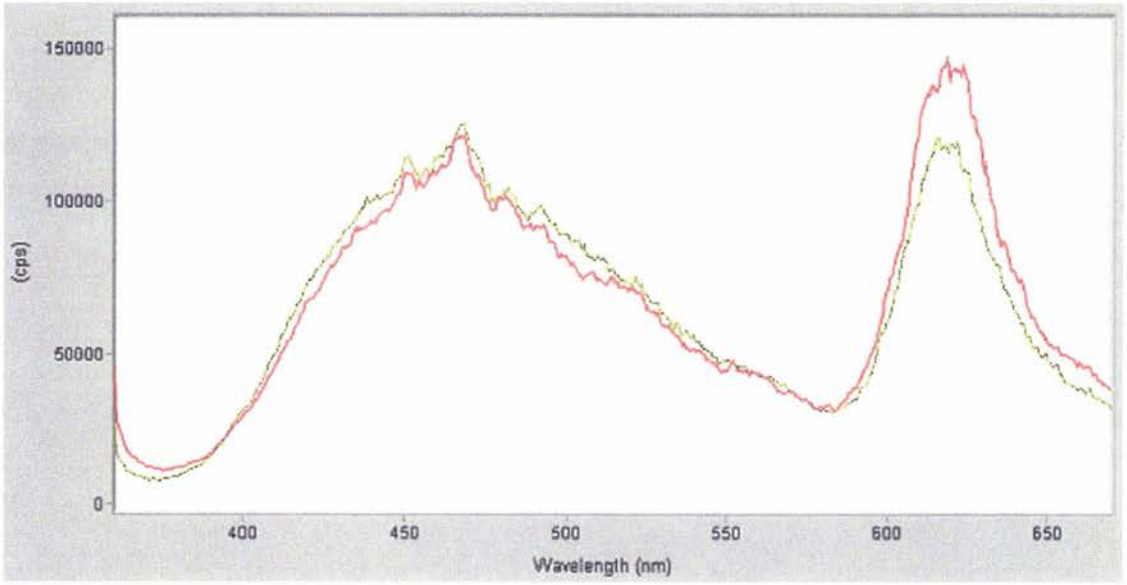


Figure 22: Emission spectrum of Texas Red Nanospheres and CdS particles, with and without Neomycin-10u-ATP CLAMP 612. Emission spectrum (excitation 360 nm) of 0.75 mg Texas Red nanospheres and 2.25 mg CdS particles in 1 ml of aptamer binding buffer was compared before CLAMP addition (red), with 45 pmol (50 ul) CLAMP (green), 90 pmol (100 ul) CLAMP (blue), and 117 pmol (130 ul) CLAMP (purple).

(a)



(b)

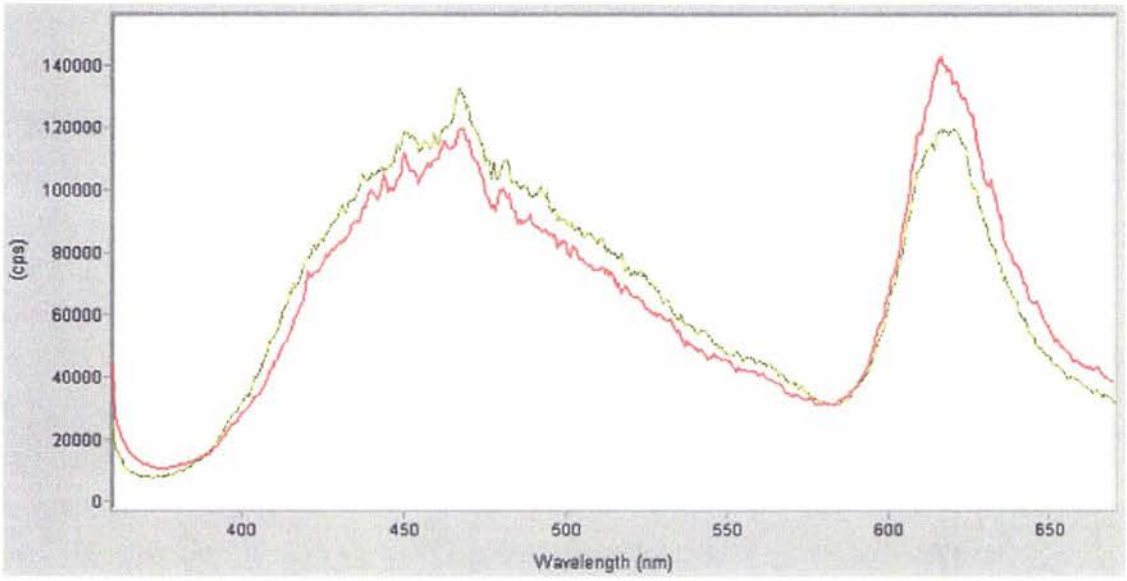


Figure 23: Emission spectrum of Texas Red Nanospheres and CdS particles with and without unpurified (a) Neomycin-uu-ATP CLAMP 609 or (b) ATP-uu-Neomycin CLAMP 610. Emission spectrum (excitation 351 nm) of 0.9 mg Texas Red nanospheres and 1.4 mg CdS particles in 2 ml aptamer binding buffer (green) was compared with the spectrum after addition of 100 ul CLAMP (red).

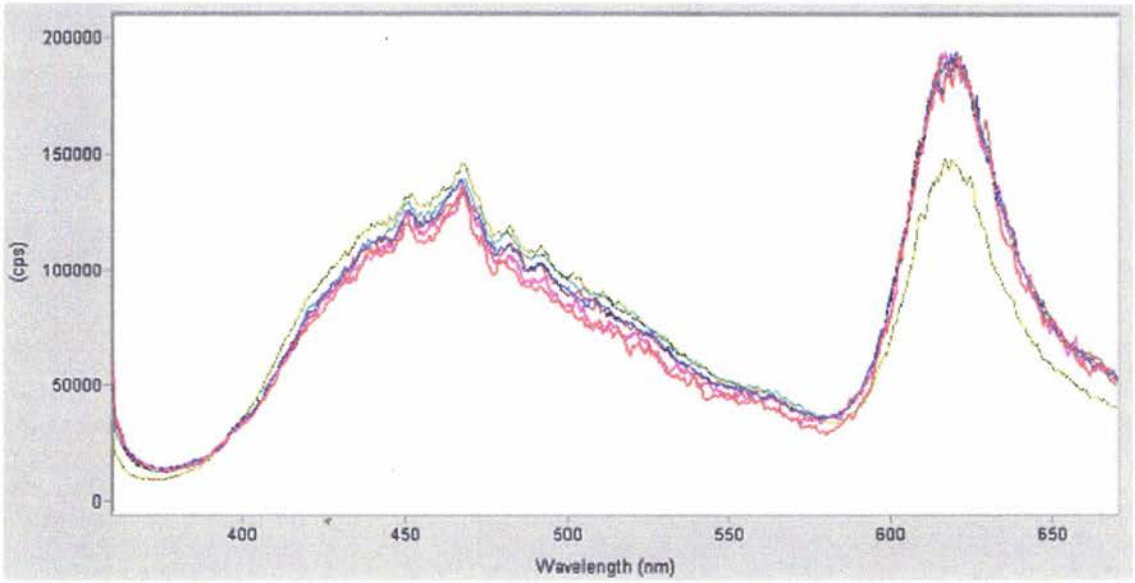


Figure 24: Emission spectrum of Texas Red Nanospheres and CdS particles with and without unpurified Neomycin-10u-ATP CLAMP 612. Emission spectrum (excitation 351 nm) of 0.9 mg Texas Red nanospheres and 1.4 mg CdS particles in 2 ml aptamer binding buffer (green) was compared with the spectrum after addition of 20 ul (aqua), 40 ul (dark purple), 60 ul (dark blue), 80 ul (light purple), and 100 ul (red) of CLAMP 612.

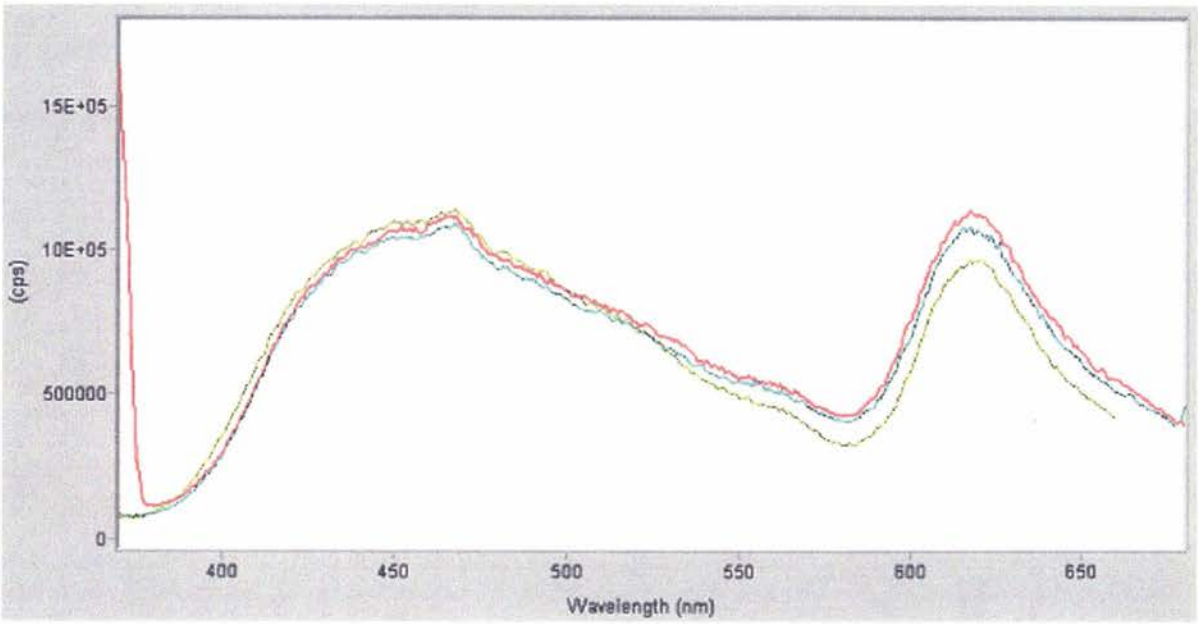


Figure 25: Emission spectrum of Texas Red Nanospheres and CdS particles with and without purified Neomycin-uu-ATP CLAMP 609. Emission spectrum (excitation 351 nm) of 0.8 mg Texas Red nanospheres and 2.0 mg CdS particles in 2 ml aptamer binding buffer (green) was compared with the spectrum after addition of 600 pmol (100 ul) of purified CLAMP 609 (aqua). Excitation at 360 nm of the final Texas Red nanospheres, CdS particles, and CLAMP 609 mixture gave slightly higher emission intensities (red).

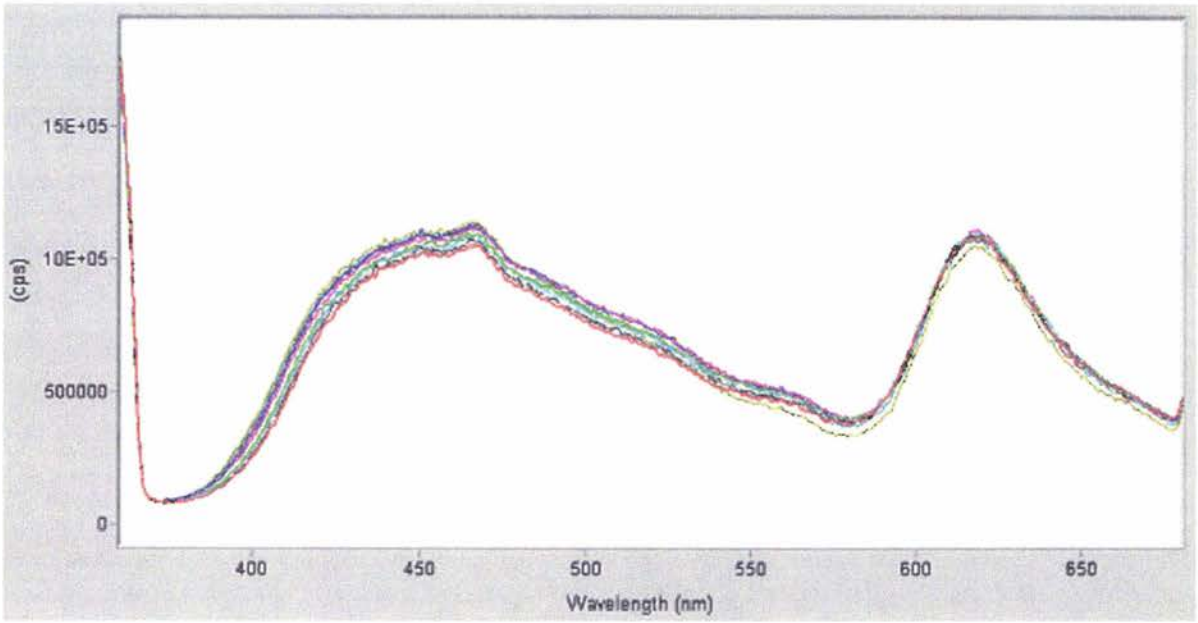


Figure 26: Emission spectrum of Texas Red Nanospheres and CdS particles with and without purified ATP-uu-Neomycin CLAMP 610. Emission spectrum (excitation 351 nm) of 0.8 mg Texas Red nanospheres and 2.0 mg CdS particles in 2 ml aptamer binding buffer (green) was compared with the spectrum after addition of 60 pmol (aqua), 120 pmol (dark purple), 180 pmol (blue), 240 pmol (light purple), 300 pmol (fluoro green), 360 pmol (fluoro blue), 420 pmol (gray), 480 pmol (dark grey), 540 pmol (black), and 600 pmol (red) of purified CLAMP 610. Total volume of CLAMP solution added was 100 ul.

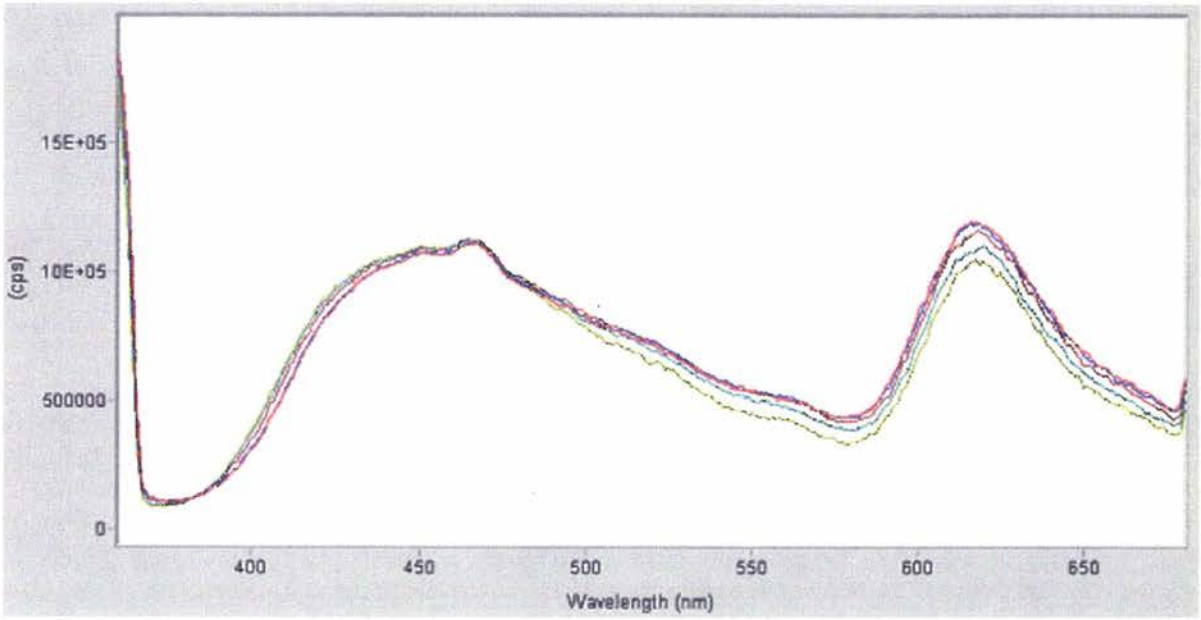


Figure 27: Emission spectrum of Texas Red Nanospheres and CdS particles with and without purified Neomycin-10u-ATP CLAMP 612. Emission spectrum (excitation 351 nm) of 0.8 mg Texas Red nanospheres and 2.0 mg CdS particles in 2 ml aptamer binding buffer (green) was compared with the spectrum after addition of 160 pmol (aqua), 320 pmol (dark purple), 480 pmol (blue), and 640 pmol (red) of purified CLAMP 612. Total volume of CLAMP solution added was 80 ul.

Conclusion

In these early experiments, much has been learned about working with the Texas Red nanospheres and CdS particles in conjunction with aptamers. FRET donor:acceptor ratio balance was improved to maximize FRET potential. The experimental design also benefitted from some optimization in order to clearly determine if the CLAMPs are producing any FRET by binding Texas Red nanospheres and CdS nanoparticles together. Although successful induction of FRET was not produced, the results presented here were a vital step in developing this new technology. The CLAMPs are capable of binding both their ligands separately, so it only remains to optimize the system until both ligands can be bound simultaneously by the same CLAMP molecule, within the distance required to produce FRET from our fluorescence donor/acceptor system.

Recommendations for Future Research

Some issues still need to be resolved before the project can move forward. The fact that the nanospheres and CdS particles do not stay in suspension without constant stirring is a problem because stirring the mixture may hinder aptamer binding to the derivatized ligands. The solvation effects also make interpreting the results difficult, since this can mimic increased Texas Red emission that may be caused by FRET. If the nanospheres and nanoparticles can be made more soluble, working with this system would be easier.

Another current unknown is the molecular weight of the nanospheres and CdS particles. Without this knowledge, the molar ratio of nanospheres or nanoparticles:CLAMP in these experiments is impossible to calculate. There are probably far more nanospheres present

in these experiments than CLAMP molecules, so the concentration of CLAMP may be severely limiting in our attempts to induce FRET. New methods are available for drastically increasing the amount of CLAMP transcribed, so increasing the concentration of CLAMP and decreasing the amount of nanospheres and CdS particles in the mixture may help balance these components until a noticeable FRET can be generated.

Aminoglycosides also appear to be less than ideal ligands for this system. These molecules are somewhat large and difficult to chemically link at the precise location desired, and they bind to many different sequences of nucleic acids, which may interfere with our CLAMPs. The CLAMP system should be moved away from using aminoglycosides as ligands, to more amenable small molecules such as ATP or theophylline.

Acknowledgement

This project was a collaboration with Victor Lin's group in the chemistry department of ISU, and all the nanosphere production and characterizations are theirs, along with the original idea of the logic gates. Daniela R. Radu produced the derivatized Texas Red nanospheres and CdS particles used here, and provided the knowledge of FRET and the fluorophores involved to get the fluorescence emission experiments running. Victor Lin designed the original project parameters and provided valuable interpretations of results as well as helpful suggestions. I wish to thank everyone who helped us get this project off the ground.

CHAPTER 4: GENERAL CONCLUSIONS

General Discussion

Aptamers are becoming a prominent molecular tool in many different disciplines. The binding affinity and specificity that make these nucleic acid molecules rival antibodies allow aptamers to be used for many applications beyond the usefulness of antibodies. Further progress in developing aptamer technology will undoubtedly depend both upon specific design of applications for aptamers as well as basic research about their behavior.

In Chapter 2 aminoglycosides restored ATP binding ability to an RNA aptamer within a CLAMP containing an additional aptamer sequence. Neomycin caused about a 2-fold increase in ATP binding of the CLAMPs, and tobramycin also showed a similar but smaller effect. CLAMPs containing either a neomycin or a theophylline aptamer in addition to the ATP aptamer were susceptible to this allosteric enhancement of ATP binding. Aminoglycosides may function as chaperones for RNA folding, potentially removing misfolding options from the dynamic folding equilibrium of these multiple aptamer constructs. This would explain the restoration of ATP binding in CLAMPs to levels consistent with the RNA ATP aptamer alone.

Chapter 3 showed the preliminary results from the application of aptamers to developing optical logic gates. Texas Red nanospheres derivatized with adenosine and CdS nanoparticles derivatized with neomycin were used as a potential FRET pair, with Neomycin/ATP CLAMPs to link the two species of nanoparticles together. FRET was not produced, but the experimental system was further developed to optimize potential for FRET

in future experiments. This system has great potential as a proof of principle to show that multiple aptamer constructs may be capable of binding several nanoparticle-sized ligands together. When this is possible, other applications for aptamers can be developed, including targeted delivery of drugs to a specific site of action in a patient's body.

Recommendations for Future Research

There is obviously much more that can be done to elucidate the behavior of aptamers and develop new technologies to use them. For the study of aminoglycoside effects upon dual aptamer constructs, other pairs of aptamers could be tested to support the broad allosteric enhancement seen in these results. Ideally, both aptamers in these constructs should be tested, to see if aminoglycosides enhance binding at both ends of a dual aptamer construct. The concentration of each aminoglycoside required to produce this effect could also be more thoroughly examined. Other aminoglycoside molecules besides the ones studied here could also be tested to determine how broadly this class of molecules affects aptamer constructs.

Much more remains to be done to develop the logic gate application of aptamer technology. The exact size and amount of the nanospheres still needs to be determined, and the ratio of CLAMP:nanosphere must be optimized. Simultaneous binding of both aptamer ligands must be obtained within the distance limits to produce FRET. One step that can be taken to simplify this first step of producing FRET will be to link a fluorescent acceptor molecule to a single aptamer sequence, which will then be used to bind the donor CdS particles. In this way, there will only be need of one aptamer to bind, and less distance constraints to obtain FRET.

REFERENCES

1. Ellington, A. D., and Szostak, J. W. (1990) *Nature 346*, 818-822.

2. Tuerk, C., and Gold, L. (1990) *Science 249*, 505-510.

3. Nishikawa, F., Kakiuchi, N., Fanaji, K., Fukuda, K., Sekiya, S., and Nishikawa, S. (2003) *Nucleic Acids Research 31*, 1935-1943.

4. Cassiday, L. A., and Maher, L. J., III. (2001) *Biochemistry 40*, 2433-2438.

5. Cassiday, L. A., and Maher, L. J., III. (2003) *Proceedings of the National Academy of Sciences 100*, 3930-3935.

6. Green, L. S., Jellinek, D., Jenison, R., Östman, A., Heldin, C.-H., and Janjic, N. (1996) *Biochemistry 35*, 14413-14424.

7. Pietras, K., Östman, A., Sjöquist, M., Buchdunger, E., Reed, R. K., Heldin, C.-H., and Rubin, K. (2001) *Cancer Research 61*, 2929-2934.

8. Bock, L. C., Griffin, L. C., Latham, J. A., Vermaas, E. H., and Toole, J. J. (1992) *Nature 355*, 564-566.

9. Padmanabhan, K., Padmanabhan, K. P., Ferrara, J. D., Sadler, J. E., and Tulinsky, A. (1993) *Journal of Biological Chemistry 268*, 17651-17654.

10. Wang, K. Y., Krawczyk, S. H., Bischofberger, N., Swaminathan, S., and Bolton, P. H. (1993) *Biochemistry 32*, 11285-11292.

11. Tasset, D. M., Kubik, M. F., and Steiner, W. (1997) *Journal of Molecular Biology 272*, 688-698.

12. Boncler, M. A., Koziolkiewicz, M., and Watala, C. (2001) *Thrombosis Research 104*, 215-222.

13. Fan, P., Suri, A. K., Fiala, R., Live, D., and Patel, D. J. (1996) *Journal of Molecular Biology 258*, 480-500.

14. Jiang, F., Kumar, R. A., Jones, R. A., and Patel, D. J. (1996) *Nature 382*, 183-186.

15. Lin, C. H., and Patel, D. J. (1996) *Nature Structural Biology 3*, 1046-1050.

16. Zimmermann, G. R., Jenison, R. D., Wick, C. L., Simorre, J.-P., and Pardi, A. (1997) *Nature Structural Biology 4*, 644-649.

17. Robertson, S. A., Harada, K., Frankel, A. D., and Wemmer, D. E. (2000) *Biochemistry 39*, 946-954.

18. Hermann, T. (2002) *Biochimie 84*, 869-875.

19. Walter, F., Putz, J., Giege, R., and Westhof, E. (2002) *EMBO J. 21*, 760-768.

20. Vuyisich, M., and Beal, P. A. (2002) *Chemistry & Biology 9*, 907-913.

21. Soukup, G. A., DeRose, E. C., Koizumi, M., and Breaker, R. R. (2001) *RNA 7*, 524-536.

22. Araki, M., Okuno, Y., Hara, Y., and Sugiura, Y. (1998) *Nucleic Acids Research 26*, 3379-3384.

23. Tang, J., and Breaker, R. R. (1998) *Nucleic Acids Research 26*, 4214-4221.

24. Jose, A. M., Soukup, G. A., and Breaker, R. R. (2001) *Nucleic Acids Research 29*, 1631-1637.

25. Tang, J., and Breaker, R. R. (1997) *Chemistry & Biology 4*, 453-459.

26. Robertson, M. P., and Ellington, A. D. (2000) *Nucleic Acids Research 28*, 1751-1759.

27. Soukup, G. A., Emilsson, G. A. M., and Breaker, R. R. (2000) *Journal of Molecular Biology 298*, 623-632.

28. Levy, M., and Ellington, A. D. (2002) *Chemistry & Biology 9*, 417-426.

29. Fourmy, D., Recht, M. I., and Puglisi, J. D. (1998) *Journal of Molecular Biology 277*, 347-362.

30. Fourmy, D., Yoshizawa, S., and Puglisi, J. D. (1998) *Journal of Molecular Biology 277*, 333-345.

31. Carter, A. P., Clemons, W. M., Brodersen, D. E., Morgan-Warren, R. J., Wimberly, B. T., and Ramakrishnan, V. (2000) *Nature 407*, 340-348.

32. Cashman, D. J., Rife, J. P., and Kellogg, G. E. (2001) *Bioorganic & Medicinal Chemistry Letters 11*, 119-122.

33. Lynch, S. R., and Puglisi, J. D. (2001) *Journal of Molecular Biology 306*, 1037-1058.

34. Kaul, M., and Pilch, D. S. (2002) *Biochemistry 41*, 7695-7706.

35. Vicens, Q., and Westhof, E. (2002) *Chemistry & Biology 9*, 747-755.

36. Faber, C., Sticht, H., Schweimer, K., and Rösch, P. (2000) *Journal of Biological Chemistry 275*, 20660-20666.

37. Cho, J., and Rando, R. R. (1999) *Biochemistry 38*, 8548-8554.

38. Tok, J. B.-H., Cho, J., and Rando, R. R. (1999) *Biochemistry 38*, 199-206.

39. Mikkelsen, N. E., Johansson, K., Virtanen, A., and Kirsebom, L. A. (2001) *Nature Structural Biology 8*, 510-514.

40. Varani, L., Spillantini, M. G., Goedert, M., and Varani, G. (2000) *Nucleic Acids Research 28*, 710-719.

41. Jin, E., Katritch, V., Olson, W. K., Kharatisvili, M., Abagyan, R., and Pilch, D. S. (2000) *Journal of Molecular Biology 298*, 95-110.

42. Hyun Ryu, D., and Rando, R. R. (2001) *Bioorganic & Medicinal Chemistry 9*, 2601-2608.

43. Jiang, L., and Patel, D. J. (1998) *Nature Structural Biology 5*, 769-774.

44. Jiang, L., Majumdar, A., Hu, W., Jaishree, T. J., Xu, W., and Patel, D. J. (1999) *Structure 7*, 817-827.

45. Wallis, M. G., von Ahsen, U., Schroeder, R., and Famulok, M. (1995) *Chemistry & Biology 2*, 543-552.

46. Cowan, J. A., Ohyama, T., Wang, D., and Natarajan, K. (2000) *Nucleic Acids Research 28*, 2935-2942.

47. Kwon, M., Chun, S. M., Jeong, S., and Yu, J. (2001) *Molecules & Cells 11*, 303-311.

48. Arya, D. P., Coffee, R. L., Jr., Willis, B., and Abramovitch, A. I. (2001) *Journal of the American Chemical Society 123*, 5385-5395.

49. Hertweck, M., Hiller, R., and Mueller, M. W. (2002) *European Journal of Biochemistry 269*, 175-183.

50. Hermann, T., and Westhof, E. (1998) *Journal of Molecular Biology 276*, 903-912.

51. Schroeder, R., Waldsich, C., and Wank, H. (2000) *EMBO 19*, 1-9.

52. Zuker, M. (2003) *Nucleic Acids Research 31*, 3406-3415.

53. Zuker, M. (accessed June 25, 2003) *Zuker group at Rensselaer Polytechnic Institute - Mfold server version 2.3* www.bioinfo.rpi.edu/applications/mfold/old/rna/form1-2.3.cgi.

54. Sassanfar, M., and Szostak, J. W. (1993) *Nature 364*, 550-553.

55. Jenison, R. D., Gill, S. C., Pardi, A., and Polisky, B. (1994) *Science 263*, 1425-1429.

56. Lai, C.-Y., Trewyn, B. G., Jeftinija, D. M., Jeftinija, K., Xu, S., Jeftinija, S., and Lin, V. S.-Y. (2003) *Journal of the American Chemical Society 125*, 4451-4459.

57. Lin, V. S.-Y., Lai, C.-Y., Huang, J., Song, S.-A., and Xu, S. (2001) *Journal of the American Chemical Society 123*, 11510-11511.

58. Lin, V. S.-Y., Radu, D. R., Han, M.-K., Deng, W., Kuroki, S., Shanks, B. H., and Pruski, M. (2002) *Journal of the American Chemical Society 124*, 9040-9041.

ACKNOWLEDGEMENTS

This work was supported by grants through the Institute for Physical Research and Technology and the Department of Energy through the Ames Laboratory.